Effects of prebiotics on the infective potential of Listeria monocytogenes

Ebersbach, Tine

Publication date:
2010

Document Version
Publisher's PDF, also known as Version of record

Effects of prebiotics on the infective potential of *Listeria monocytogenes*

Ph.D. Thesis

by

Tine Ebersbach

The National Food Institute

May, 2010

Technical University of Denmark

DTU

Preface

The present thesis is submitted in order to fulfill the requirements for obtaining the Ph.D. degree at The National Food Institute, Technical University of Denmark.

Most of the work presented here was carried out at The National Food Institute under guidance of Professor Tine Rask Licht whom I wish to thank for enthusiastic guidance, inspiration and support during this project. Also thanks to my co-supervisor Senior Scientist Morten Poulsen for his advice and help during this project. I wish to thank Professor Robert W. Hutkins at the Department of Food Science and Technology at University of Nebraska, Lincoln, USA for accepting me as a guest in his laboratory during the spring of 2009 and all the people in his laboratory for making me feel so welcome.

I would also like to thank Doctor Sampo Lahtinen and Doctor Arthur Ouwehand from Danisco Finland, Professor Hanne Frøkiær, University of Copenhagen and Professor Peter M. H. Heegaard, Technical University of Denmark for fruitful collaboration.

I also want to thank the animal facility at The National Food Institute for assistance during the animal experiments and technician Bodil Madsen and Kate Vibefeldt for help in the laboratory.

I am grateful to all my colleagues at the Department of Microbiology and Risk Assessment for creating an enjoyable working atmosphere. A very special thanks go to "the girls from the basement-office" for rewarding scientific discussions as well as for all the fun we have had together.

Finally, a warm thanks to my family and friends for their endless patience and support.

May 2010

Tine Ebersbach

Summary

The human gastrointestinal tract is colonized by a dense and complex community of bacteria. The intestinal microbiota has a large impact on the health of the host and the intestinal bacteria are roughly classified as either potential deleterious or potential beneficial bacteria. Several factors can affect the composition of the microbiota - among them prebiotics. Prebiotics are food ingredients that are non-digestible for the human body and therefore reach the large intestine in an intact form. In the large intestine the prebiotics selectively stimulate the growth of the beneficial rather than the harmful bacteria of the microbiota.

Gastro-intestinal infections currently cause several hundred thousand reported cases of disease in the EU each year. Infections with the forborne pathogen *Listeria monocytogenes* are relatively rare, but it is one of the most severe infections in the industrialised countries with a mortality of about 30%.

The gut has a very important function in defending the host against infections with ingested pathogenic bacteria and there is increasing evidence that prebiotics can help strengthen this defense. This is done through stimulation of beneficial intestinal bacteria that release bacteriocins toxic for the pathogens, lower the pH to a level that is unfavourable for pathogenic bacteria and compete with the pathogen for nutrients and mucosal adhesion sites in the intestine. However, besides the microbiota dependent mechanisms increasing evidence suggest that prebiotics exert their protective function against pathogens through microbiota independent mechanisms. This is thought to be done by blocking the pathogenic adhesion to intestinal cells, affecting the expression of virulence genes from the pathogen and by stimulating the immune system.

In vivo evidence of the prebiotics effect against pathogenic enteric infections is scarce and I therefore investigated the effect of five non-digestible carbohydrates (putative prebiotics) on the resistance of guinea pigs to infection with three different strains of *L. monocytogenes*. Animals were fed a diet supplemented with either 10% xylooligosaccharides (XOS), galactooligosaccharides (GOS), inulin, apple pectin or polydextrose for three weeks before oral challenge with *L. monocytogenes*. XOS and GOS significantly improved resistance of guinea pigs to *L. monocytogenes,* while inulin and apple pectin decreased the resistance. No significant effect on resistance to *L. monocytogenes* was seen after feeding with polydextrose. To further explore the mechanisms behind these *in vivo* observations, microbiota independent effects of

four of the carbohydrates (XOS, GOS, inulin and polydextrose) on the adhesive and infective potential of *L. monocytogenes* was investigated. Mixing *L. monocytogenes* with XOS just prior to infection decreased the adherence of two of the three strains of *L. monocytogenes* to the intestinal cell line Caco-2. Additionally, 2 hours incubation with XOS and subsequently washing of the bacteria decreased the adherence of all three strains of *L. monocytogenes* to Caco-2 cells. No effect on adhesion was seen for either GOS, inulin or polydextrose.

Adherence to the intestinal epithelium is considered a very important step in the infection cycle for most of the pathogenic bacteria. Without adherence the pathogenic bacteria are rapidly eliminated from the intestine. The ability of the four carbohydrates to affect the expression of *L. monocytogenes* genes known to be involved in adherence to intestinal cells (*inlA, lap, ami, iap, aut, fdpA, actA*) was therefore investigated. It was found that expression of the adhesion genes was affected in a strain dependent manner by the presence of prebiotics in the growth media.

In conclusion, these results show that different non-digestible carbohydrates can have entirely different effects on the *in vivo* infectivity of *L. monocytogenes* and that microbiota independent mechanisms might be involved. All the tested carbohydrates affected expression of adherence genes but only XOS affected the *in vitro* adhesion of *L. monocytogenes* to intestinal cell. This may suggest that different mechanisms are responsible for the observed *in vivo* effect of the different non-digestible carbohydrates. Mostly microbiota independent mechanisms were investigated in this project, but it is very likely that microbiota dependent mechanisms also are involved.

Dansk sammendrag

Den menneskelige mave-tarmkanal er koloniseret af et tæt og kompleks samfund af bakterier. Tarmens mikrobiota har en stor indvirkning på sundheden hos værten og tarmbakterier kan groft klassificeret som enten potentielt skadelige eller potentielt gavnlige. Flere faktorer kan påvirke sammensætningen af mikrobiotaen – herunder prebiotika. Prebiotika er fødevare komponenter, der ikke kan nedbrydes i den menneskelige gastrointestinale kanal og derfor når tyktarmen i intakt form. I tyktarmen stimulerer prebiotika hovedsageligt de gavnlige frem for skadelige bakterier i mikrobiotaen.

Hvert år bliver flere hundrede tusinde mennesker, alene i EU, syge af mavetarm infektioner. Infektioner med den fødevarebårne patogene bakterie *Listeria monocytogenes* er forholdsvis sjældne, men med en dødelighed på omkring 30% er det en af de mest alvorlige infektioner i de industrialiserede lande.

Tarmens microbiota har en meget vigtig funktion i at beskytte værten mod infektioner med patogene bakterier fra maden, og flere og flere beviser tyder på at prebiotika kan medvirke til at styrke denne beskyttelse. Dette sker ved stimulering af de gavnlige tarmbakterier der derved frigiver bakteriociner der er giftige for de patogene bakterier, sænker pH til et niveau der er ugunstig for de patogene bakterier og konkurrence med de patogene bakterier om næringsstoffer og adhæsions receptorer i tarmen. Ud over de mikrobiota afhængige mekanismer er der stadig flere tegn på, at prebiotika kan udøve sin beskyttende funktion mod patogene bakterier gennem mikrobiota uafhængige mekanismer. Dette menes at ske ved blokering af de patogene bakteriers adhæsions receptorer, påvirkning af ekspression af virulens gener og ved at stimulere immunforsvaret.

Indtil nu er der kun udført få *in vivo* infektionsforsøg med prebiotika, og derfor har jeg undersøgt hvilken effekt fem ikke-fordøjelige kulhydrater (formodede prebiotika) har på marsvins modstandsdygtighed overfor tre *L. monocytogenes* stammer. Dyrene blev i tre uger fodret med en kost suppleret med enten 10% xylooligosaccharider (XOS), galactooligosaccharider (GOS), inulin, æble pektin eller polydextrose, før de blev oralt inficeret med *L. monocytogenes*. XOS og GOS forbedrede marsvinenes modstandsdygtighed overfor *L. monocytogenes* infektionen, mens inulin og æble pektin fik modstandsdygtigheden til at falde. Der var ingen effekt efter fodring med polydextrose. For yderligere at udforske de mekanismer der ligger til grund for *in vivo* observationerne, blev fire af kulhydraternes (XOS, GOS, inulin og polydextrose) mikrobiota

uafhængige virkninger på infektionspotentialet af *L. monocytogenes* undersøgt. Ved tilsætning af XOS lige inden infektion faldt adhæsionen til tarm cellelinjen Caco-2 af to af de tre *L. monocytogenes* stammer. To timers inkubation med XOS og efterfølgende vask af *L. monocytogenes* bakterierne fik også adhæsionen til Caco-2 cellerne af alle tre stammer til at falde. Hverken GOS, inulin eller polydextrose havde effekt på adhæsionen af *L. monocytogenes in vitro*.

Adhæsion til tarm epitelet betragtes, for de fleste patogene bakterier, som et meget vigtigt trin i infektionscyklussen. Uden adhæsion vaskes de patogene bakterier meget hurtigt ud af tarmen. Derfor blev de fire kulhydraters påvirkning af ekspressionen af syv gener (*inlA, lap, ami, iap, aut, fdpA, actA*) der menes at være involveret i adhæsion af *L. monocytogenes* til tarm epitelet undersøgt. Dette viste at ekspressionen af adhæsionsgenerne blev påvirket på en stamme afhængig måde af tilstedeværelse af kulhydraterne.

Ud fra disse resultater kan det konkluderes, at forskellige ikke-fordøjelige kulhydrater kan have helt forskellige virkninger på *in vivo* infektiviteten af *L. monocytogenes*, og at mikrobiota uafhængige mekanismer kan være involveret. Alle de testede kulhydrater påvirkede ekspressionen af adhæsionsgenerne, mens kun XOS påvirkede adhæsionen af *L. monocytogenes* til epitel cellerne. Dette kan tyde på, at det er forskellige mekanismer, der er ansvarlig for de observerede *in vivo* effekter af de forskellige ikke-fordøjelige kulhydrater. Hovedsageligt mikrobiota uafhængig mekanisme er blevet undersøgt i dette projekt, men det er meget sandsynligt, at mikrobiota afhængige mekanismer også kan være involveret.

Objectives of the study

Foodborne gastrointestinal infections are a serious problem affecting public health worldwide. Gastro-intestinal infections currently cause several hundred thousand reported cases of disease alone in the EU each year. From a health and socioeconomic perspective the distress, possible medical treatment, hospitalization and loss of working power following a gastrointestinal infection presents a serious problem. Listeriosis caused by the foodborne pathogen *Listeria monocytogenes* is a very severe disease with a mortality rate of 20-30% despite fast treatment with antibiotics (Vazquez-Boland *et al.*, 2001). Listeria infections are relatively rare, however, a reduction in the occurrence is very important given the high mortality rate. For unknown reasons Denmark has for a number of years had the highest occurrence of listeriosis in the EU (EFSA Annual Report, 2007).

The aim of this study was to utilize guinea pigs to identify dietary prebiotic carbohydrates inhibiting infections with *L. monocytogenes* and to further explore the mechanisms behind this expected preventive effect. The intension was to analyze what impact feeding of prebiotic had on the intestinal microbiota of guinea pigs and to compare it to the observed *in vivo* effects. I therefore isolated feacal DNA from guinea pigs before and after feeding with prebiotics and sent it to BGI-Shenzhen, China to obtain metagenome information by Solexa sequencing. However, I did not receive any data from China so I instead decided to investigate the direct interactions between prebiotics, *Listeria monocytogenes* and the intestinal epithelium.

The first part of this thesis gives a theoretical introduction to *Listeria monocytogenes,* the gastrointestinal tract and prebiotics. The second part contains two manuscripts where my work constitutes the core. I have furthermore contributed in the making of two manuscripts: "Response of *Listeria monocytogenes* to oxygen restriction: A microarray-based transcription analysis" and "Characterization of serum amyloid A as an acute phase protein in the guinea pig". However, as the focus of these two manuscripts is in the periphery of the scope of my Ph.D. work these manuscripts are not included in the thesis.

List of manuscripts

Ebersbach, T., Jørgensen, J. B., Heegaard, P. M., Lahtinen, S. J., Ouwehand, A. C., Poulsen M, Frøkiær, H., Licht, T. R. (2010) Certain dietary carbohydrates promote *Listeria* infection in a guinea pig model, while others prevent it. International Journal of Food Microbiology. *140*, 218-224.

Ebersbach, T., Andersen J. B., Bergström. A., Hutkins R. W., Licht, T. R. Effect of prebiotics on the infective potential of *Listeria monocytogenes*. (2010) Submitted to International Journal of Food Microbiology.

Not included in this thesis

Andersen, J. B., Bergström, A., Hansen T. B., Larsen, L. S., Knudsen G. M., **Ebersbach T**., Christensen B. B., Boye, M., and Licht, T. R. Response of *Listeria monocytogenes* to oxygen restriction: A microarray-based transcription analysis. Paper in preparation

Heegaard, P.M., **Ebersbach, T**., Licht, T. R. (2010) Characterization of serum amyloid A as an acute phase protein in the guinea pig. Paper in preparation.

Table of contents

Theoretical Part

Listeria monocytogenes

Listeria

The gram-positive bacterial genus *Listeria* consists of a group of facultative intracellular and facultative anaerobic bacteria. *Listeria* are very widely distributed in nature and can be found in many environmental sources including feces from humans and animals, soil, water and in a large variety of foods (Vazquez-Boland *et al.*, 2001). *Listeria* is acquired by eating contaminated food products and can cause acute, self limited gastroenteritis in otherwise healthy individuals (Ooi and Lorber, 2005) and the more severe invasive disease listeriosis in immunocompromised patients. Although *Listeria* has been isolated from nearly all kinds of food, food most often contaminated is raw products consumed without prior cooking or reheating. In the small intestine *Listeria* enters cells of the intestinal epithelium and pass in that way through the intestinal barrier. The *Listeria* bacteria are carried via the blood to the liver which is the main site of replication. In severe cases, *Listeria* infects the central nervous system leading to meningitis or infects the placenta of pregnant women leading to infections of the fetus (Sleator *et al.*, 2009).

The genus *Listeria* includes six species of which only two species *Listeria monocytogenes* (*L. monocytogenes*) and *L. ivanovii* are potentially pathogenic to humans (Vazquez-Boland *et al.*, 2001). Human infections with *L. ivanovii* (predominantly infects ruminants) are very rare (Cummins *et al.*, 1994;Guillet *et al.*, 2010) and *L. monocytogenes* account for almost all reported cases of human listeriosis (EFSA Annual Report, 2007). Listeriosis is usually a very severe disease with a mortality rate of 20-30% despite fast treatment with antibiotics. Human infections with *Listeria* are relatively rare (0.3 cases per population of 100,000 in the EU in 2007) yet important to investigate given the severity of symptoms and the high mortality rate. For unknown reasons Denmark has for a number of years had the highest notification rate in EU with 1.1 cases of listeriosis per population of 100,000 (EFSA Annual Report, 2007).

Survival in the gastrointestinal tract

The pathophysiology of *L. monocytogenes* infection in humans and animals is still poorly understood. Most of our knowledge is a result of observations from experimental infections of animals and interpretation of epidemiological and histopathological findings (Mead *et al.*, 1999).

Inside the body *L. monocytogenes* has to withstand a lot of adverse environments before it transiently can colonize the intestine and invade the body. In the stomach, *L. monocytogenes* has to survive the acidic conditions. Treatment of patients or animals with antacids has shown to reduce resistance to *L. monocytogenes* indicating that the acidity of the stomach destroys a significant number of *L. monocytogenes* (Donnelly, 2001). It is known that moderately acidic conditions (pH 5-5.5) can start an adaptive response that can enhance survival of *L. monocytogenes* under conditions of otherwise lethal pH (pH 3.5) (Davis *et al.*, 1996;O'Driscoll *et al.*, 1996). This stress response might increase survival in the gastrointestinal (GI) tract and later within the macrophage phagosome.

A similar "stress hardening" response is thought to be started when *L. monocytogenes* is grown under anaerobic conditions. Andersen *et al* (2007) showed that oxygen restriction increased the infective potential of *L. monocytogenes* both *in vitro* and *in vivo* in oral infection assays of guinea pigs. Unpublished results suggest that this increase in infectivity is due to an increased expression of genes known to be important for *L. monocytogenes* adhesion and invasion of epithelial cells (Andersen, personal communication).

Passage from the stomach to the small intestine involves an osmotic upshift for *L. monocytogenes*. To counterbalance this high turgor pressure *L. monocytogenes* transports osmolytes into the bacterial cell and in that way prevents water loss from the bacteria (Gahan and Hill, 2005).

Once in the small intestine *L. monocytogenes* also has to tolerate the bactericidal activities of bile. Several genes are involved in this response however, one gene, bile salt hydrolase (*bsh*) is of particular importance. *bsh* is absent from nonpathogenic *Listeria* species and it has been shown that *bsh* is involved in the persistence of *L. monocytogenes* in the small intestine of oral infected guinea pigs (Dussurget *et al.*, 2002). Rather surprisingly, despite the high concentration of bile *L. monocytogenes* is able to colonize the gall bladder of mice (Hardy *et al.*, 2004). This is found to be a result of the neutral pH in the gall bladder making *L. monocytogenes* more resistant to high concentrations of bile (Begley *et al.*, 2005).

Adherence to and invasion of the gastrointestinal mucosa

L. monocytogenes has multiple strategies to invade a wide range of mammalian cells. *L. monocytogenes* can be engulfed by professional phagocytes such as M-cells, however, the main way of entrance into hosts normally susceptible to *L. monocytogenes* is for the bacteria to "force" their own entry into otherwise non-phagocytic cells such as intestinal enterocytes and hepatocytes (Lecuit *et al.*, 2001;Corr *et al.*, 2006).

The initial crossing of the intestinal barrier by *L. monocytogenes* is obviously very crucial in initiating the infection and during the years a lot of research has been done in this field. Galliard *et al* (1991) showed that the protein Internalin A (InlA) is responsible for the invasion of *L. monocytogenes* into epithelial cell lines and in 2001 the importance of InlA for adherence and invasion of *L. monocytogenes* was confirmed *in vivo* (Lecuit *et al.*, 2001). However, because a very rapid translocation to internal organs is seen after inoculation of rats and mice with *L. monocytogenes* (Pron *et al.*, 1998;Daniels *et al.*, 2000) it was long speculated that *L. monocytogenes* also used a rapid non-specific transport system to cross the intestinal barrier. This was confirmed by Corr *et al* (2006) which showed that *L. monocytogenes*, without involving the classical virulence factors, can colonize murine Peyers patches *in vivo* and translocate through M cells *in vitro*.

Even though, it is generally believed that InlA is the primary protein involved in infection of enterocytes by *L. monocytogenes,* evidence suggest that yet other proteins may be involved in this process. How big a role these proteins play in the crossing of the intestinal barrier is far from elucidated because experiments are few and mainly done *in vitro*. For some of these proteins namely the autolysins their role might be fine tuning of the surface architecture of *L. monocytogenes* priming the bacteria for adherence and invasion of enterocytes. The role for other proteins might be to mediate a first unspecific binding between *L. monocytogenes* and the host cell bringing the two cells so close that a second host/cell specific binding can take place. And yet other proteins involved in the adherence may have a completely different way of function (Milohanic *et al*., 2001). I will in the following section describe Internalin A and some of the proteins believed to be important for the adherence and invasion of *L. monocytogenes* into enterocytes.

Internalin A

L. monocytogenes primarily enters mammalian cells through the action of two surface proteins Internalin A (InlA) and InlB (Gaillard *et al.*, 1991). InlA is attached to the cell surface while InlB only is loosely associated to the bacterial surface and also exit as a released soluble form (Khelef *et al.*, 2006). The *inlA* and *inlB* genes are only found in *L. monocytogenes* strains and not in the non-pathogenic *L. innocua* strains (Gaillard *et al.*, 1991;Glaser *et al.*, 2001). InlA are believed to be the principal protein involved in uptake of *Listeria* into enterocytes (Lecuit *et al.*, 2001) and it is also believed to play a critical role in the crossing of the placenta (Lecuit *et al.*, 2001;Disson *et al.*, 2008). InlA binds to the epithelial receptor E-cadherin (Mengaud *et al.*, 1996) which is located below the tight junctions between polarized epithelial cells (Pentecost *et al.*, 2006). Normally, InlA have no access to E-cadherin but during extrusion of apoptotic cells at the villous tips E-cadherin is exposed to the luminal surface. It has been shown that *L. monocytogenes* takes advantage of this process to adhere to E-cadherin (Pentecost *et al.*, 2006). Binding of InlA to E-cadherin promotes a complex host signaling cascade and reorganization of the cytoskeleton in the enterocytes leading to internalization of *L. monocytogenes* (Bonazzi *et al.*, 2009). Latex beads coated with InlA or expression of InlA by the normally non-invasive *L. innocua* is sufficient to promote phagocytosis into epithelial cell lines *in vitro* (Lecuit *et al.*, 1997;Pron *et al.*, 1998). Studies have shown that if a *Listeria* strain contains a truncated *inlA* gene the efficiency of internalization into cultured cell lines will be low (Jonquieres *et al.*, 1998). In continuation of this it has been shown that a significantly higher number of clinical *Listeria* strains involved in bacteremia contains full length InlA compared to *Listeria* strains isolated from food (Jacquet *et al.*, 2004). Also epidemiological studies have shown that *L. monocytogenes* strains with full length InlA are associated with isolates from pregnancy-related cases (Jacquet *et al.*, 2004;Disson *et al.*, 2008). This clearly shows the importance of InlA for the virulence of *L. monocytogenes*.

InlB is required for the systemic spread of *L. monocytogenes* and is important for the invasion of hepatocytes and placenta (Disson *et al.*, 2008). Until recently it was not possible to demonstrate a role for InlB in the intestinal phase of infection. However, a very recent study suggests that after *L. monocytogenes* has attached to the intestinal epithelium InlB activates its receptor Met. This leads to an acceleration of the internalization of *L. monocytogenes* by increasing the endocytosis of junctional components (Pentecost *et al*., 2010).

Listeria adhesion protein

Listeria adhesion protein (LAP) is one of the proteins that have been shown to be involved in adhesion of *L. monocytogenes* to intestinal cells (Pandiripally *et al.*, 1999;Jaradat *et al.*, 2003). Deletion of *lap* does not seem to have any effect on the adhesion of *L. monocytogenes* to non-intestinal cell lines e.g. from the liver, kidney and bladder (Jaradat *et al.*, 2003). After oral administration of mice with either a wild-type (wt) strain or a LAP-deficient strain a significantly higher number of wt *L. monocytogenes* was found in the liver. Intraperitoneal infection of mice showed no difference between the two strains suggesting that the LAP-deficient strain was unable to cross the intestinal barrier (Jaradat *et al.*, 2003). LAP is identified as an alcohol acetaldehyde dehydrogenase, which in other bacteria is involved in adherence to eukaryotic cells (Kim *et al.*, 2006). LAP is found mainly in the cytoplasm, with only a few proteins (10-15%) located on the cell membrane (Jaradat and Bhunia, 2002). Expression of *lap* is induced by nutrient-limitation (Jaradat and Bhunia, 2002), temperature around 37°C (Santiago *et al.*, 1999) and anaerobicity (Burkholder *et al.*, 2009) - all factors found in the GI tract. The heat shock protein 60 (Hsp60) has been shown to act as a receptor for LAP (Wampler *et al.*, 2004). Hsp60 is a mitochondrial chaperone, however, it has also been found in the membrane in a variety of cell types, including intestinal epithelial cells (Soltys and Gupta, 1997).

Fibronectin-binding protein A

Human fibronectin is a glycoprotein found in plasma and extracellular fluids and as an insoluble form in the extracellular matrix (Gilot et al., 1999). Fibronectin is involved in a number of eukaryotic cellular processes but is also known to work as an attachment protein for several pathogenic bacteria (Courtney et al., 1994;Fowler et al., 2000). *L. monocytogenes* binds to fibronectin via fibronectin-binding protein A (FdpA) and infectious studies with a fdpA deletion strain in transgenic mice have shown a significantly lower number of *L. monocytogenes* in the intestine, lymph nodes and liver compared to infections with a wt strain (Gilot et al., 2000;Dramsi et al., 2004). In oral infections of mice not having a functional receptor for InlA (see paragraph "Animal models used for infection studies with *L. monocytogenes*") no difference in the number of L. monocytogenes in the intestine and organs are seen between the two strains (Dramsi et al., 2004). This indicates that FdpA is dependent on the InlA-E-cadherin interaction to perform its function in helping *L. monocytogenes* cross the intestinal barrier.

ActA

The surface protein ActA is a major virulence factor of *L. monocytogenes* mediating actin-based intra- and intercellular spread (Smith *et al.*, 1995). However, it has suggested that ActA also is implicated in epithelial cell invasion (varez-Dominguez *et al.*, 1997). The ability of *L. monocytogenes* to adhere to several eukaryotic cell lines, among them intestinal cell lines, is impaired if ActA is deleted, and furthermore, expression of ActA enables *L. innocua* to invade epithelial cells (Suarez *et al.*, 2001). ActA binds to the ubiquitously distributed human heparin sulfate proteoglycan and this binding might be involved in ActA function in adhesion of *L. monocytogenes* (varez-Dominguez *et al.*, 1997;Suarez *et al.*, 2001). Further studies need to be done to elucidate what role ActA plays in the adhesion process of *L. monocytogenes.*

Ami

Bacterial autolysins are endogenous enzymes that digest the cell wall peptidoglycan of their own cell walls. These molecules have been implicated in various biological functions, such as regulation of cell growth, cell division and protein secretion (Popowska, 2004).

In 1997 Braun *et al*. (1997) identified Ami, a protein with autolytic activity exclusively found on the surface of *L. monocytogenes*. A significant loss of adherence to eukaryotic intestinal cell lines *in vitro* is seen if *ami* is inactivated in an *inlA* and/or *inlB* mutant (Milohanic *et al.*, 2000;Milohanic *et al.*, 2001). However, this loss of adhesion is not seen in a wt strain, suggesting that InlA and InlB are able to overcome the defect in Ami mediated adhesion (Milohanic *et al.*, 2001). In mice, intravenously infected with either an *ami* deletion strain or a strain where both *ami*, *inlA* and *inlB* are deleted fewer *L. monocytogenes* are seen of the two deletions strains in the liver compared to infection with a wt strain. This indicates that Ami might also play a role after the translocation of the epithelial tissue (Milohanic *et al.*, 2001). The disruption of *ami* only affects the adhesion of *L. monocytogenes* and not the ability to grow once *L. monocytogenes* is internalised into the eukaryotic cell (Milohanic *et al.*, 2000). The C-terminal domain of Ami is believed to be responsible for the attachment of *L. monocytogenes* to eukaryotic cells and if *ami* is inactivated in an *inlA* and/or *inlB* background the expression of this domain fully restores the adhesion (Milohanic *et al.*, 2001).

p60

The protein p60 (gene name *iap*) is another autolysin also believed to be involved in the adherence of *L. monocytogenes* to intestinal cells (Kuhn and Goebel, 1989). p60 is found both on the cell surface and as a secreted protein and is required for proper cell division of *L. monocytogenes* (Kuhn and Goebel, 1989;Ruhland *et al.*, 1993). Free p60 directly binds to intestinal epithelial cells with high specificity (Park *et al.*, 2000) correlating with several studies showing that in the absence of p60 the invasiveness of *L. monocytogenes* into intestinal epithelial cell is reduced (Pilgrim *et al.*, 2003;Faith *et al.*, 2007). Once inside the epithelial cell the p60 mutant escapes from the phagosome and grows in the cytosol with the same efficiency as a wt stain. However, the deletion mutant fails to produce actin tails, which results in reduced intracellular movement and reduced cell-to-cell spread (Pilgrim *et al.*, 2003). After intragastrically infection of mice with a *L. monocytogenes* p60 mutant, significantly fewer *L. monocytogenes* bacteria are found in caecum and in the internal organs (Faith *et al.*, 2007). The fact that fewer bacteria are present in the caecum might indicate that p60 is required for maximal virulence of *L. monocytogenes* already in the gastrointestinal tract.

Auto

Auto is yet another *L. monocytogenes* autolysin involved in entry of *L. monocytogenes* to intestinal cells (Cabanes *et al.*, 2004). Auto is essential for entrance of *L. monocytogenes* into several cell lines among them intestinal cell lines. Fewer Listeria bacteria were found in the intestine and organs after oral infection of guinea pigs. Similarly, fewer Listeria bacteria were found in the organs of mice after intravenous infection with an *auto* deletion strain compared with infection with a wt strain (Cabanes *et al.*, 2004). This implies that Auto is involved both in the intestinal phase and in later stages of the infectious process. Bublitz *et al*., (2009) suggests that Auto might be involved in coordinating the release of virulence factors at the right stages of infection.

Animal models used for infection studies with *L. monocytogenes*

Previously, mice have been the most extensively used animal model to study the pathophysiology of *L. monocytogenes* and the immune response to *L. monocytogenes*. However,

observations showing that in murine cell lines, no difference in entry was observed between *L. monocytogenes* and InlA mutant led to the discovery that InlA exhibit species specificity for some E-cadherins (Lecuit *et al.*, 1999). Interaction between InlA and E-cadherin is completely impaired in mouse and rats whereas it is functional in a lot of other mammalian species e.g. guinea pigs, rabbits, humans and gerbils (Lecuit *et al.*, 1999;Disson *et al.*, 2008) (see figure 1). A proline in position 16 of E-cadherin was shown to be responsible for this species specificity. Substitution of this proline with glutamic acid as seen in the E-cadherin of mouse and rat completely abrogate interaction between InlA and E-cadherin (Lecuit *et al.*, 1999).

Not all of the symptoms of a *L. monocytogenes* infection in guinea pigs and rabbits are similar to those of human listeriosis. Infections in guinea pigs and rabbits do not lead to an efficient infection in the central nervous system, which is often seen in humans (Gray and Killinger, 1966). Inspired by the discovery of the species specificity of InlA and E-cadherin, Khelef *et al.* (2006) found that species specificity was also involved in the binding of InlB to its receptors Met (Shen *et al.*, 2000) and gClq-R (Braun *et al.*, 2000). InlB is unable to interact with its receptors in guinea pig and rabbit and therefore it cannot promote entry (Khelef *et al.*, 2006) (see figure 1).

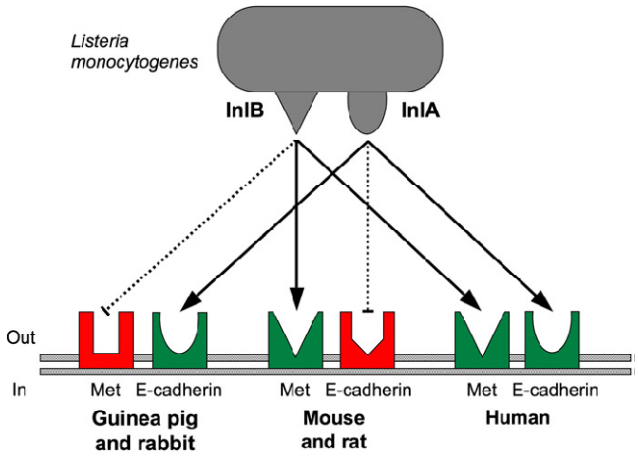


Figure 1: Species specificity between InlA and E-cadherin and between InlB and Met. Reprinted from (Lecuit, 2007).

These species specificities clearly show that all of the above described animal models have their drawbacks in mimicking human listeriosis. However, these models can be used as long as you carefully keep in mind the limitations of the model and what questions you want to answer.

Now, novel animal models have been developed to study listeriosis after oral inoculation. Two genetically engineered mouse lines have been developed. The first was a transgenic mouse line where human E-cadherin was exclusively expressed in the enterocytes of the small intestinal (Lecuit *et al.*, 2001). The disadvantage of this model is that the role of interaction between InlA and E-cadherin in other tissue than the small intestine (e.g. the colon, caecum, placenta and the blood-brain barriers) cannot be assessed. Recently, a new genetically engineered mouse line has been developed that account for this tissue problem. In this knock-in E16P mouse line the glutamic acid at position 16 of the endogenous mouse E-cadherin has been replaced with a proline resulting in binding between InlA and the mouse E-cadherin (Disson *et al.*, 2008).

Intracellular life cycle of *L. monocytogenes*

After entrance into the host cell either by phagocytosis into professional phagocytotic cells or by induced phagocytosis into non-phagocytosis cells *L. monocytogenes* undergoes a characteristic replication cycle (figure 2) (Hamon *et al*., 2006). This cycle starts by interaction between the plasma membrane and *L. monocytogenes* causing the plasma membrane to enwrap the bacteria by a so called "zipper" mechanism (figure 2a) (Swanson and Baer, 1995;Mengaud *et al*., 1996). After penetration of the cell membrane the *L. monocytogenes* bacteria become engulfed within a phagocytic vacuole, which shortly after becomes acidified (figure 2b). Already two hours after entry about 50% of the *L. monocytogenes* bacteria are free in the cytoplasm where they multiplies (figure 2c). An actin tail is formed at one of the poles of the bacteria leading to random movement in the cytoplasm (figure 2d). Some of the *L. monocytogenes* bacteria reach the cell membrane and make a finger-like protrusion that can penetrate neighbouring cells (figure 2e). Inside the new cell, the bacteria are engulfed in a secondary double-membrane phagosome of which the bacteria quickly escapes and a new replication cycle can begin (figure 2f) (Hamon *et al*., 2006).

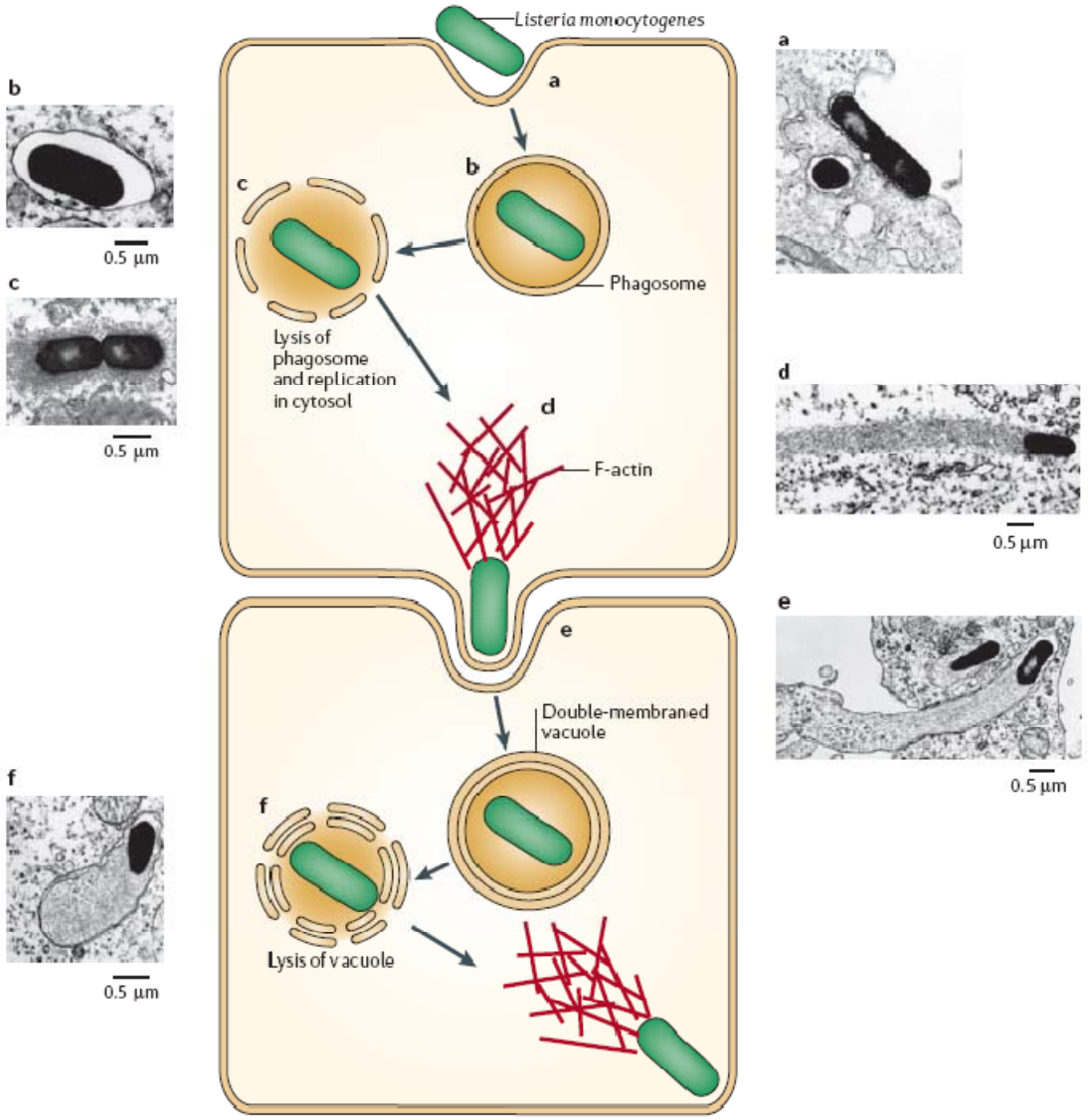


Figure 2: Schematic representation and electron micrographs of the different stages of the intracellular life cycle of *L. monocytogenes.* a: Adhesion to and penetration of the eukaryotic cell. b: Survival within the phagocytic vacuole (phagosome). c: Escape from the phagosome and replication in the cytosol. d: Actin-based motility. e: Actin polymerization allows *L. monocytogenes* to move in the cytosol and form a finger-like protrusion leading to penetration of neighbouring cells. f: Escape from the double-membraned secondary phagosome and beginning of a new replication cycle. Reprinted from (Hamon *et al.*, 2006).

Carbohydrates and gene expression in *L. monocytogenes*

The ability of *L. monocytogenes* to sense differences in the environment and hereafter to conduct an appropriate gene response may be crucial for the success as a pathogen. One of the best

known examples of this is the temperature-dependent control of Positive regulatory factor A (PrfA) which is the master virulence regulator of *L. monocytogenes*. A posttranscriptional thermoregulation ensures that PrfA and with that most of the virulence genes, only are expressed at temperatures found inside a host (Leimeister-Wachter *et al.*, 1992;Johansson *et al.*, 2002).

Another example of the ability of *L. monocytogenes* to act according to the environment is the repression of virulence genes in response to certain fermentable carbohydrates that *L. monocytogenes* may find outside a host (Park and Kroll, 1993;Park, 1994;Milenbachs *et al.*, 1997;Gilbreth *et al.*, 2004;Larsen *et al.*, 2006). This ability seems in some degree to be strain dependent (Park and Kroll, 1993;Milenbachs *et al.*, 1997). The exact mechanism behind this carbon mediated repression of virulence genes has not been elucidated yet but the virulence genes are only repressed by carbohydrates that can be readily metabolized by *L. monocytogenes* and only when present in amounts able to enhance growth (Premaratne *et al.*, 1991;Milenbachs *et al.*, 1997). However, it should be noted that growth enhancement by a carbohydrate is not in itself enough to repress the virulence genes (Milenbachs *et al.*, 1997). It cannot be ruled out that the carbohydrates in itself function as a signaling molecule although it is more likely that the repression of virulence genes are under a more global control, such as catabolite repression (Milenbachs *et al.*, 1997;Gilbreth *et al.*, 2004).

The gastrointestinal tract

The human gastrointestinal tract

The major structures of the human gastrointestinal (GI) tract are: the oral cavity, esophagus, stomach, small intestine, colon, rectum and anus. The small intestine is composed of the duodenum, the jejunum and the ileum while the caecum, ascending colon, transverse colon, descending colon and sigmoid rectum make up the colon (Kutchai, 1998) (see Figure 3).

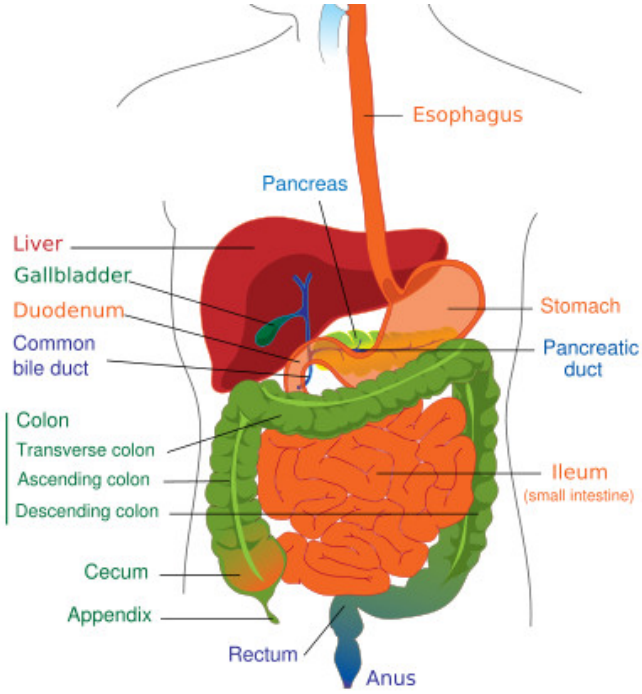


Figure 3: Schematic representation of the human gastrointestinal system.

The GI tract plus the associated organs, salivary glands, liver, gallbladder and pancreas, make up the GI system. The major function of the GI system is to digest ingested food and absorb the nutrients into the body. The digestion and absorption of nutrients depends greatly upon movements of the GI tract which soften, and mix the GI content with secretions from the

associated glands and move it along the tract. The secretion of substances into the GI content is very important for degradation of food particles into molecules that can be absorbed by the body. Interplay between hormones, paracrine molecules and neurons regulates the function of the GI tract (Kutchai, 1998).

Digestion of carbohydrates

The major sources of carbohydrates in the human diet are polysaccharides (e.g. starches and nonstarch polysaccharides), disaccharides (e.g. sucrose and lactose) and monosaccharides (e.g. glucose and fructose) (Kutchai, 1998;Roberfroid, 2005c). Most carbohydrates have to be digested in the GI tract before absorption since epithelial cells only absorb carbohydrates in the form of monosaccharides.

Most of the digestion and absorption of carbohydrates takes place in the small intestine with relatively low activity in the duodenum, high activity in the jejunum and then again low activity in the ileum (Roberfroid, 2005b). However, the first digestion of starch already begins in the mouth where α-amylase from the saliva catalyzes the hydrolysis of the internal α-1.4 linked polymer of glucose into oligo- and di-saccharides. The digestion by α-amylase continues until the enzyme is inactivated by the gastric acid in the stomach. In the duodenum the acid pH is neutralized and the highly active pancreatic α-amylase takes over the process of digestion (Despopoulos and Silbernagl, 1991). Further digestion is done by other enzymes both from the pancreatic juice and from the mucosa. The end product of monosaccharides is taken up by the mucosal cells. Human intestinal enzymes can only hydrolyze α-glycosidic bonds (with the exception of lactase that hydrolyze β-glycosidic linkages between glucose and galactose molecules in lactose) thus cellulose and other molecules with β-glycosidic linkages arrive undigested to the colon. Here the undigested carbohydrates are fermented by the microbiota producing short chain fatty acids (SCFAs), gases and biomass. Some fermentation of the β-glycosidic linked molecules is also done by the microbiota in the small intestine (Kutchai, 1998;Roberfroid, 2005c).

It is not only β-glycosidic linked molecules that arrive undigested to the colon. Usually not all of the ingested starch is completely digested and absorbed in the small intestine. Some is therefore passed on to the colon where it is fermented by the colonic microbiota (Kutchai, 1998).

Composition of the human gastrointestinal microbiota

The human gastrointestinal tract is colonized by a dense and complex community of bacteria having a large impact on health of the host via e.g. conversion of metabolites, protection against pathogens and effect on the immune system and on colonic health (Vaughan *et al.*, 2002). The gut is a nutrient-rich, open system with a constant temperature and flow. Because of this constant flow, organisms in the gut either have to attach to or colonize host tissues or to reproduce at a rate sufficient to avoid washout (Flint *et al.*, 2007).

The total estimate of microorganisms in the GI tract is approximately 10^{14}. However, the actual number of bacteria differs greatly among the different regions of the GI tract with only 10^3 colony forming units (cfu) per gram content in the stomach, 10^4-10^7 cfu per gram in the small intestine and 10^{10}-10^{12} cfu per gram in the colon where the density of bacteria is highest (Holzapfel *et al.*, 1998). Approximately 60% of feces is composed of bacteria (Stephen and Cummings, 1980).

Previously, the composition of the microbiota has been elucidated through culturing techniques, however, many microbial species cannot be cultured because their preferred growth conditions are unknown or difficult to reproduce. Thus, research on microbial diversity is increasingly making use of new culture-independent techniques that classify bacteria based upon phylogenetic comparison of 16S rRNA sequences (Flint *et al.*, 2007;Bik, 2009). Very recently the human microbiota of 124 European adults have been examined by metagenomic sequencing (Qin *et al.*, 2010). By using these culture-independent techniques far more phylotypes have been found. Nevertheless, it is still believed that we have not characterized all of the normal human microbiota yet (Rajilic-Stojanovic *et al.*, 2007). Up to now, around 1000 distinct microorganisms (bacteria species, as well as archaea and eukarya) have been found in the human GI microbiota (Rajilic-Stojanovic *et al.*, 2007;Boesten and de Vos, 2008;Qin *et al.*, 2010). Despite this enormous biodiversity, the majority of the human-associated bacteria are members of only four phyla, namely *Firmicutes* (including the large class of *Clostridia* and the lactic acid bacteria), *Bacteroidetes*, *Actinobacteria* (including *Collinsella* and *Bifidobacterium* spp.), and *Proteobacteria.* Bacteria from the two phyla *Firmicutes* and *Bacteroidetes* account for the majority of the bacteria in the colon (Dethlefsen *et al.*, 2007;Qin *et al.*, 2010). Subdominant groups are enterobacteriacae, streptococci, and lactobacilli. The human microbiota is dominated by gram-positive and aerobic bacteria (Guarner and Malagelada, 2003;Boesten and de Vos, 2008).

The composition of the human microbiota is comparable to the microbiota of other mammals at the phylum level while most bacterial families and genera seem to be distinct (Dethlefsen *et al.*, 2007).

The balanced microbiota

The microbiota has a significant impact on the health of the host and an "unbalance" in the composition of the microbiota is thought to be a contributory factor in the development of several diseases e.g. acute gastroenteritis, colon cancer and more chronic disorders such as inflammatory bowel disease (Gibson and Fuller, 2000). Some of the colonic bacteria are thought to be potentially deleterious (e.g. *Clostridia* spp., *Staphylococci* spp. and *Proteus* spp.) and some potentially beneficial (e.g. *Bifidobacterium* spp. and *Lactobacillus* spp.) but the classification as either harmful or beneficial is often hard to make, both because of limited knowledge and because some bacteria genera seem to have ambiguous physiological functions (figure 4) (Roberfroid, 2008a).

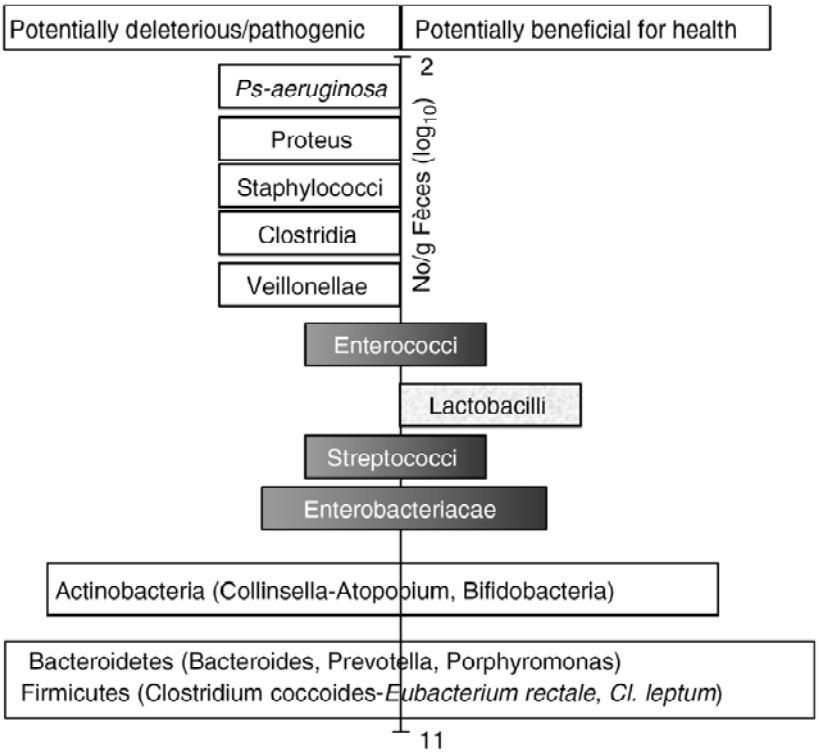


Figure 4: Schematic average distribution of components of human fecal microbiota divided between potentially deleterious and potentially beneficial groups. Some of the bacteria groups are still difficult to

classify as either deleterious or beneficial because of lack of knowledge. Preliminary results suggest that at least some members of the groups *Eubacterium rectal* and *Cl. Leptum* may be classified as beneficial (Roberfroid, 2008a).

Positive effects have been attributed to the presence of *Bifidobacterium* and *Lactobacillus* predominantly present in the colon and ileum, respectively (Boesten and de Vos, 2008). Abundance of bifidobacteria and lactobacilli in the human microbiota has e.g. been implicated in protection against gastrointestinal infections and allergies (Bruzzese *et al.*, 2006). Even though most research has been done on bifidobacteria and lactobacilli also other bacteria are believed to be beneficial to the host. *Faecalibacterium prausnitzii* for instance has been found to be underrepresented in patients with inflammatory bowel disease (Manichanh *et al.*, 2006). Recently, Sokol *et al*. (2008) found that *F. prausnitzii* has anti-inflammatory properties and this mechanism may explain the reversed relationship between the presence of *F. prausnitzii* and inflammatory bowel disease.

The composition of the normal so-called balanced microbiota is still insufficiently described and the physiological impact of some of the different genera is uncertain. The difficulties that have been in distinguishing the "good" bacteria from the "bad" might reflect the wide variety of interactions between different bacteria groups in the microbiota. Because of the large degree of cross-feeding and other interactions between bacteria groups some of the bacteria now considered harmful are maybe, in small numbers, necessary for maintaining health and well being of the host (Roberfroid, 2005d).

Individual variation and age related changes in the human microbiota

The major dominant bacteria groups as described above are almost always present in all individuals, but considerable species variations between individuals are common (Eckburg *et al.*, 2005;Wells *et al.*, 2008). Qin *et al*., (2010) found by metagenomic sequencing a core of species of approximately 6% common to over 50% of the individuals tested.

During birth the establishment of the intestinal microbiota is started. The infant composition of the microbiota quickly changes during the first few weeks and new bacteria species establish in the GI tract during the first years of life. After two years the microbiota begins to resemble the microbiota of an adult (Boyle and Tang, 2006). It is usually aerobic or facultative anaerobic

bacteria, like enterobacteria, enterococci and staphylococci that are the first to establish in the infant gut. These bacteria consume the oxygen making the way for the proliferation of anaerobic bacteria like *Bifidobacterium*, *Clostridium* and *Bacteroides*. As the oxygen concentration in the gut drops the number of aerobic bacteria decline (Adlerberth, 2008). Several studies have been made on what influence feeding mode has on the microbiota of infants, however, the literature is often contradictory. In spite of that there is general agreement that numbers of Clostridium (especially *C. difficile*) are lower in breast-fed infants as compared with formula-fed infants (Vael and Desager, 2009).

Although the composition of the young and adult microbiota is thought to be quite stable several factors can influence the composition of the microbiota, among them the environment, age, gender and diet (Eckburg *et al.*, 2005;Wells *et al.*, 2008). The number and species diversity of beneficial bacteria such as anaerobic bacteroides and bifidobacteria are declining with age in conjunction with an increased number of facultative anaerobes (Mitsuoka *et al.*, 1974;Woodmansey *et al.*, 2004;Bartosch *et al.*, 2004). These changes might help explain the decreased gut functionality often seen in elderly people.

Anaerobic fermentation of carbohydrates by the human microbiota

The majority of colonic bacteria use carbohydrates not already digested and absorbed in the small intestine as their main feeding substrate. In the ascending part of the colon where the concentration of non-digested carbohydrates is highest the metabolism of carbohydrates is quantitatively more important than amino acid fermentation. Because of the increased fermentation the pH in this part of the colon is often lower than in the rest of the colon (Roberfroid, 2005b;Flint et al., 2007). The most frequently used substrates for colonic carbohydrate fermentation are non-starch or plant cell wall polysaccharides (including cellulose, pectin and polymers of e.g. glucose or xylose), oligosaccharides (e.g. inulin-fructans) and starches that have not been fully digested in the small intestine (Roberfroid, 2005b). The main fermentation end products are short chain fatty acids (SCFA) (mainly acetate, propionate and butyrate), ethanol, gases and intermediate metabolites (e.g. formate, lactate and succinate). Around 95% of the SCFA produced in the colon is absorbed by the intestinal epithelial cells (Cummings and Macfarlane, 1991) where they serve as energy sources for colonic epithelial

cells (especially butyrate), anti-inflammatory agents and regulators of gene expression. SCFAs therefore have a significant effect on the gut environment and on the host (Roberfroid, 2005b).

The breakdown of carbohydrates in the colon is a complex process involving different groups of bacteria via metabolic cross-feeding. One group of bacteria may be able to degrade a complex polysaccharide resulting in the release of oligosaccharides that they do not utilize. These molecules can now be further digested by other groups of bacteria (Rossi *et al.*, 2005).

The gastrointestinal tract and microbiota of guinea pigs

The anatomy of the gastrointestinal (GI) tract of guinea pigs is relatively well known. The major difference between the GI tract of humans and guinea pigs is the large caecum of the guinea pig (see figure 5).

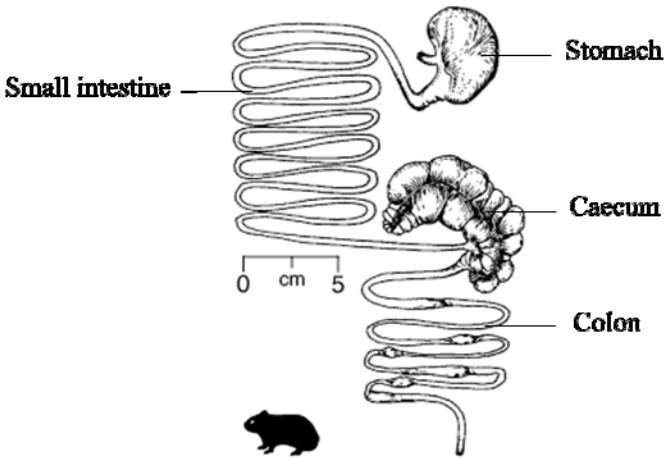


Figure 5: Schematic representation of the gastrointestinal system of a guinea pig.

The caecum of rodents is a large thin-walled sack located in the crossing between the small intestine and the colon (Breazile and Brown, 1976). The caecum of humans is rudimentary and does not make a bulge and is simply the first part of the colon where the appendix is fixed (Kutchai, 1998). The caecum of rodents and guinea pigs is the major site of fermentation especially of plant materials such as cellulose. Humans mainly carry out fermentation in the colon (Kararli, 1995). This fermentation leads to a large production of SCFA and it has been estimated that up to 44% of the energy requirement of animals like guinea pigs with a well-

developed caecum come from SCFA while the energy derived from SCFA in humans only account for 6-9% (McNeil, 1984;Leser and Molbak, 2009). Only a few studies have investigated the composition of the microbiota of guinea pigs (Erichsen, 1969;Yanabe *et al*., 2001;Takahashi *et al*., 2005). The caecal microbiota of guinea pigs has among others species been shown to include species of Bacteroidaceae, Eubacteria, Clostridia, Bifidobacteria, Lactobacillus and Enterobacteriaceae (Yanabe *et al*., 2001).

Prebiotics

Pre- and probiotics

The composition of the human gut microbiota can be affected by the diet. The increased knowledge of the human microbiota and of the considerable impact it has on the health of the host has led to the development of dietary components that can sustain or improve the human microbiota. Probiotics are one of such dietary components developed to positively affect the GI microbiota. Probiotics are viable beneficial microorganisms, often bifidobacteria or lactobacilli, presently found in many food items such as yogurt, juice or capsules (Rijkers *et al.*, 2010).

Prebiotics are another type of dietary component aiming at producing a beneficial effect on the GI microbiota. Prebiotics are dietary compounds that are nondigestible for the human body and therefore reach the large intestine in an intact form. In the large intestine the prebiotics are fermented by preferable the beneficial rather than the harmful bacteria of the microbiota (Bruzzese *et al.*, 2006). Gibson *et al.* (2004) defined a prebiotic as "a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microbiota that confers benefits upon host well-being and health". The focus of this section of the thesis will be on prebiotics.

Established and putative prebiotics

Some clear criteria need to be fulfilled for a carbohydrate to be called a prebiotic: 1. Resistance to gastric activity and resistance to hydrolysis by mammalian enzymes 2. No absorption in the upper gastrointestinal tract 3. Fermentation by the intestinal microbiota 4. Selective stimulation of the growth and/or activity of intestinal bacteria believed to be beneficial to the host (Gibson *et al*., 2004). The fourth criterion demonstrating selectivity is the most difficult to fulfill. Experiments are most correctly done *in vivo*, all though *in vitro* assays with fecal innocua often gives a fairly good picture of the selectivity of the carbohydrate tested (Gibson *et al*., 2004;Roberfroid, 2008b). As seen from the criteria listed above not all nondigestible carbohydrates are prebiotics, however, many food oligosaccharides and polysaccharides have

incorrectly been claimed to be prebiotics without meeting the criteria (Roberfroid, 2007). So far only two food ingredients i.e. inulin-type fructans and galactooligosaccharide fulfill all the criteria to be called prebiotics (Roberfroid, 2007). In the following section inulin and galactooligosaccharide (also called trans-galactooligosaccharide) and three prospective prebiotic, xylooligosaccharides (XOS), polydextrose and apple pectin will be further discussed (table 1). These are the five carbohydrates that have been used in the experimental part of this thesis.

Prebiotic	Structure	Degree of polymerization	Production	Reference
Inulin	Fructans joined by β(1→2) glycosidic bonds, often terminated by a glucose molecule	Between 2 and 60. Average DP of 12	Commonly extracted from chicory	(Roberfroid, 2005d)
Galacto-oligosaccharide	Galactose units joint by β(1→2), β(1→3) or β(1→4) linkages, often terminated by a glucose molecule.	Between 2 and 6	Enzymatic conversion of lactose. The intermediates lactose, glucose and galactose are often found in the final product	(Macfarlane *et al.*, 2008;Albrecht *et al.*, 2010)
Xylo-oligosaccharide	Xylose units joint by β(1→4) glycosidic bonds	Between 2 and 6	Degradation of xylan molecules from xylan rich agricultural residues e.g. corn cobs	(Martin-Pelaez *et al.*, 2008;Roberfroid, 2008b;Akpinar *et al.*, 2010)
Polydextrose	Randomly bonded polymers of glucose, sorbitol and citric or phosphoric acid (mostly 1→6-glycosidic bonds)	Between 1 and 100. Average DP of 12	Condensation of glucose under high temperature and partial vacuum	(Voragen, 1998;Burdock and Flamm, 1999;Zhong *et al.*, 2000)
Apple pectin	α(1→4)-linked galacturonic acid interspersed with few rhamnose units linked to galacturonate units with β(1→2) and β(1→4) bonds	Large, complex molecule. DP > 2	Commonly extracted from apple pomace.	(May, 1990;Caffall and Mohnen, 2009)

Table 1: Structure, degree of polymerization and typical production methods for inulin, galactooligosaccharide, xylooligosaccharide, polydextrose and apple pectin.

Inulin

Inulin is naturally found in a large variety of plants that uses inulin as a mean of storing energy (Roberfroid, 2005d). Inulin is resistant to hydrolysis by the human enzymes in the small intestine because the fructose monomers are linked by β(2→1) glycosidic bonds. Both *in vitro* and *in vivo* testing have confirmed that inulin reach the large intestine undigested (Roberfroid, 1993) and extensive *in vitro* culture fermentation studies and human trials have shown that inulin selectively stimulate the growth of bifidobacteria and lactobacilli (Wang and Gibson, 1993;Gibson and Wang, 1994;Bouhnik *et al.*, 1996;Tuohy *et al.*, 2001). Moreover, human trials have shown that addition of inulin and galactooligosaccharides to formula of infants results in an increase in bifidobacteria and lactobacilli (Moro *et al.*, 2002;Boehm *et al.*, 2002). This is consistent with data showing that bifidobacteria are able to break down and utilize inulin by the action of their β-fructofuranosidase enzyme (Manzanares and Hardy, 2008).

Galactooligosaccharide

Galactooligosaccharides (GOS) are oligosaccharides naturally occurring in human breast milk (Kunz *et al.*, 2000). No studies have undoubtedly shown that GOS reach the colon without being hydrolyzed by the human enzymes in the small intestine (Roberfroid, 2007). A number of *in vitro* studies and human trials investigating the influence of GOS on the microbiota have shown that GOS has a positive effect on the number of lactobacilli and in an even higher degree on the number of bifidobacteria combined with a unchanged or decreased number of enterobacteria (Minami *et al.*, 1983;Rowland and Tanaka, 1993;Ito *et al.*, 1993;Bouhnik *et al.*, 1997). As stated in the previous paragraph human trials with infants shows an increase in the fecal number of bifidobacteria when a mixture of GOS and inulin is added to the infants formula milk (Moro *et al.*, 2002;Boehm *et al.*, 2002)**.**

The convincing data from the human studies imply that GOS are considered a prebiotic (Roberfroid, 2008b).

Xylooligosaccharide

Xylooligosaccharides (XOS) are usually made by degradation of xylan rich molecules. Xylan are naturally found in e.g. birchwood, beechwood, corncob, and oat spelt (Akpinar et al., 2009). No

data exist to confirm that the xylooligosaccharides resist the digestive processes in the gut and small intestine, however, xylan is defined as a dietary fiber suggesting that it may reach the colon intact (Roberfroid, 2008b). Moreover, XOS is stable over a wide range of pH even at the relatively low pH of the gastric juice (Vazquez *et al.*, 2000). Several *in vivo* and *in vitro* studies have shown a positive stimulation of the growth of bifidobacteria by XOS but so far failed to show a convincing selective stimulation of bacterial growth (Campbell *et al.*, 1997;Jaskari *et al.*, 1998;Vazquez *et al.*, 2000;Martin-Pelaez *et al.*, 2008)

Scientific evidence still needs to be published before XOS can be regarded as a prebiotic and it will therefore be more correct to classify it as an "emerging prebiotic".

Polydextrose

Polydextrose is an artificial made polysaccharide with no natural occurrence. Polydextrose is used in the food industry as a replacement for fat and sucrose providing only 25% of the caloric value of sucrose (Voragen, 1998;Burdock and Flamm, 1999). Because of the high molecular weight and the complexity of polydextrose it is almost intact when it reaches the colon where it is partially digested by the indigenous microbiota (Burdock and Flamm, 1999).

Conflicting data exist about the effect of polydextrose on the composition of the microbiota. Probert *et al* (2004) and Hernot *et al* (2009) found by *in vitro* fermentation studies that polydextrose had positive effect on the number of bifidobacteria. Similarly, Zhong *et al* (2000) found in a human trial that intake of polydextrose increase the number of bifidobacteria and lactobacilli in fecal samples, while the number of bacteroides decrease. Contrary, Fava *et al* (2007) and Hengst *et al* (2009) found no effect of intake of polydextrose on the number of bifidobacteria and lactobacilli in pigs intestinal content and in fecal samples from humans, respectively.

Because of the lack of compelling scientific data from human trials polydextrose is not presently regarded as a prebiotic.

Apple pectin

Pectin is found in the primary cell wall of plants and is the traditional gelling agent for jams and jellies. Pectin is also used in the food industry e.g. in fruit products and dairy products (May,

1990;Caffall and Mohnen, 2009). Pectinases and pectolytic enzymes that hydrolyse pectin are widely distributed in higher plants and microorganisms. Pectins are defined as a dietary fiber and as other dietary fibers pectin is not digested in the small intestine and therefore reach the large intestine intact (Cummings and Englyst, 1987;Mandalari *et al.*, 2007). Several *in vitro* studies have shown that pectins increase the number of bifidobacteria and lactobacilli (Crociani *et al.*, 1994;Olano-Martin *et al.*, 2002;Mandalari *et al.*, 2007). Olano-Martin *et al* (2002) found that oligosaccharides derived from pectin have a greater impact on the growth of bifidobacteria than pectins. Besides the positive effect on the number of bifidobacteria and lactobacilli, pectin has also other positive physiological effects in the gastrointestinal tract. These include delayed gastric emptying and reduced glucose absorption, both characters thought to be due to the gel forming and water holding capacity of pectin (Olano-Martin *et al.*, 2002).

On the basis of the data obtained through *in vitro* fermentations, pectins exhibit potential to be classified as a prebiotic, however, human trials have to be conducted to confirm the *in vitro* data.

Prevention of gastrointestinal infections with prebiotics

Besides being the place for the initial digestion and absorption of nutrients the gut also has a very important function in defending the host against infections with ingested pathogenic bacteria. A number of studies have shown that intake of prebiotics can help to prevent infections with pathogenic bacteria. Several mechanisms appear to be involved and they can roughly be divided into microbiota-dependent and microbiota-independent mechanisms. These mechanisms will be addressed in the following section.

Microbiota dependent mechanisms

Lactic acid bacteria have been shown to protect against infection with *L. monocytogenes* both *in vitro* and *in vivo*. Thus the *in vitro* invasion of *L. monocytogenes* into intestinal cell lines has been inhibited by a number of lactic acid bacteria (Altenhoefer *et al*., 2004;Corr *et al*., 2007). Administration of live Lactobacillus strains prior to challenge with *L. monocytogenes* has been shown to significantly protect mice against infection (Bambirra *et al*., 2007;Vieira *et al*., 2008). The idea is that prebiotics help protect the host against pathogenic infections by increasing the density and metabolic activity of the beneficial bacteria of the indigenous microbiota. Several

mechanisms are thought to be involved in this microbiota-dependent protective effect of administration of prebiotics. It is believed that stimulation of the beneficial bacteria leads to an increased competition for nutrients and adhesion sites with the pathogen, increased production of SCFA and bacteriocins harmful for the pathogen and stimulation of the immune system (Bourlioux et al., 2003).

Competition for nutrients

It is a common mechanism that the bigger the indigenous microbiota of any given ecological niche the harder it will be for an outside bacterium, whether pathogenic or nonpathogenic, to establish itself in the microbiota (Wells *et al.*, 2008). Fewer nutrients will be available and this will start a strong competition where only those bacteria most efficient at utilizing the nutrients will survive. It can therefore be hypothesized that for a prebiotic to be most effective, it has to be administered in advance before the pathogen is introduced giving the prebiotic time to stimulate the growth of the indigenous bacteria (Wells *et al.*, 2008).

Bifidobacteria can grow on a range of different prebiotics (inulin, fructooligosaccharides (FOS), GOS, XOS and pectin) (Wang and Gibson, 1993;Rowland and Tanaka, 1993;Campbell *et al.*, 1997;Olano-Martin *et al.*, 2002). However, bifidobacteria are especially good at fermenting inulin and FOS. This is due to the ability of the bifidobacteria to produce β-fructofuranosidase and a special transport system that helps the bifidobacteria take up FOS with a DP below 8 (Janer *et al.*, 2004). Bifidobacteria are believed to hydrolyze inulin and FOS at the cell surface and degrade the shorter molecules intracellularly so that no intermediate products are released to the surroundings and by that to the competing bacteria (Bosscher *et al.*, 2006). The ability of pathogenic bacteria to utilize prebiotics is an area that has been given relatively little focus (Fooks and Gibson, 2002;Petersen *et al.*, 2009).

Production of short-chain fatty acids

The fermentation of prebiotics by bifidobacteria and lactobacilli in the colon leads to the production of short-chain fatty acids (SCFA) and to the production of gases (CO_2, H_2 and CH_4). The SCFA produced depend on the type of substrate fermented although the SCFA most often produced is acetate, propionate and butyrate, while lactate, ethanol, succinate, formate, valerate

and caproate are produced in smaller amounts (Mountzouris et al., 2002). SCFA in the colon affect the potential for pathogenic bacteria to induce infection in several ways. Increased production of SFCA lead to acidification of the colon making growth difficult for some pathogenic bacteria (Wang and Gibson, 1993;Fooks and Gibson, 2002;Bosscher *et al*., 2006;Louis *et al*., 2007). Lactic-acid producing bacteria are generally very acid tolerant and hence, their growth is less affected. This acidification also results in increased mucin production believed to improve mucosal morphology and by that decreasing the colonization and translocation of pathogenic bacteria (Barcelo *et al*., 2000;Lomax and Calder, 2008). SCFA are also known to affect the immune system and in that way to influence the infective potential of pathogenic bacteria (Lomax and Calder, 2008).

Production of bacteriocins

Bacteriocins are antimicrobial peptides or proteins produced by strains of diverse bacterial species in response to the presence of other bacteria. Bacteriocins have a relatively narrow killing spectrum and are often only toxic to bacteria closely related to the strain producing them (Wells *et al.*, 2008). Some of the colonic strains of bifidobacteria and lactobacilli have been found to produce bacteriocins and this may give them a competitive advantage within the complex ecosystem of the gut (Servin, 2004). Bacteriocins produced by bifidobacteria and lactobacilli have been shown to be active against pathogenic bacteria such as strains of *Listeria, E. coli, Salmonella and Clostridia* (Ferreira and Lund, 1996;Zamfir *et al.*, 1999;Casadei *et al.*, 2009;Slavica *et al.*, 2010). The mechanism of action for many bacteriocins is to form pores in the cytoplasmic membrane of the target bacteria and in that way dissipate the proton motive force. Other bacteriocins work by interfering with essential proteins of the target bacteria (Servin, 2004). Bacteriocins are nontoxic to eukaryotic cells and are generally recognized as safe substances. Comprehensive research in the use of bacteriocins as natural food preservatives is done by the food industry. Yet, the only bacteriocin used commercially is nisin produced by strains of *Lactococcus lactic* (Galvez *et al.*, 2008). Chen *et al* (2007) found in an *in vitro* assay that FOS directly induce bacteriocin production from selected strains of *Lactobacillus* and *Lactococcus*.

Competition for adhesion sites

Adherence to the intestinal cells is considered a very important step in the infection cycle for most pathogen bacteria. Without adherence the pathogenic bacteria cannot withstand the flow of the intestinal chyme and they are rapidly eliminated from the intestine (Lee *et al.*, 2003). A prebiotic stimulation of the indigenous bacteria population leading to increased occupation of intestinal adherence sites and thereby inhibition of the adherence of pathogens is therefore thought to be one of the mechanisms that underlies the positive effects of prebiotics against pathogenic bacteria. Several *in vitro* studies have shown that strains of bifidobacteria and lactobacilli can inhibit, displace and compete with the adherence of pathogens such as *L. monocytogenes, E. coli* and *S.* Typhimurium to intestinal cell lines and intestinal mucus (Tuomola *et al.*, 1999;Lee *et al.*, 2003;Collado *et al.*, 2005;Collado *et al.*, 2006).

Microbiota independent mechanisms

Most of the protective effect of prebiotics is believed to go through stimulation of beneficial bacteria from the indigenous microbiota. However, it has also been discovered that prebiotics in itself can exert some protective mechanism against infection with enteric pathogens.

Attenuation of virulence

It has been suggested that adherence inhibition by prebiotics may be due to modulation of the virulence genes of the pathogen (Knutton *et al.*, 1997;Vanmaele *et al.*, 1999;Shoaf *et al.*, 2006). Regulation of virulence genes by the presence of carbohydrates in the growth media has been seen for a number of bacteria (Ankenbauer and Nester, 1990;VanGijsegem, 1997;Brencic and Winans, 2005) including *L. monocytogenes* (Park and Kroll, 1993;Milenbachs *et al.*, 1997;Gilbreth *et al.*, 2004). The repression of virulence genes in *L. monocytogenes* could either be because the prebiotic molecules functioned as a signaling molecule *per se* or more likely be a result of catabolite repression (see paragraph "Carbohydrates and gene expression in *L. monocytogenes*").

Receptor decoy

Certain carbohydrates structurally resemble the saccharide-containing glycoproteins that many pathogens bind to on the intestinal cell (Kunz *et al.*, 2000). This has lead to the hypothesis that some carbohydrate may act as an intestinal receptor decoy for pathogenic bacteria by mimicking the host cell receptor on the intestinal cell that the pathogen normally adhere to. By binding to the carbohydrate instead of to the intestinal cells the pathogen is washed from the gastrointestinal tract and has little chance of invading the body. This has been confirmed in several *in vitro* studies where the ability of several carbohydrates to inhibit the adherence and invasion of pathogenic bacteria has been studied (Tzortzis *et al.*, 2005;Shoaf *et al.*, 2006;Rhoades *et al.*, 2006;Rhoades *et al.*, 2008;Searle *et al.*, 2009). The inhibition of adherence is strain dependent and some of these studies have shown that some carbohydrates can have no or the opposite effect of what was intended and instead enhanced the adherence (Ruas-Madiedo *et al.*, 2006;Searle *et al.*, 2009).

Microbiota dependent and independent mechanism

Immunomodulation

The intestine is the largest immune system in the body. It is estimated that the intestine contains approximately 80% of all antibody-producing cells and produces antibodies more efficiently than any other part of the body (Fukasawa *et al.*, 2007). The diet and the digestion products are in close contact with the intestinal immune system including the gut-associated lymphoid tissue (GALT). Even though data from human studies are still scarce, data from animal studies clearly indicate that the diet can modulate immune functions in multiple ways (Watzl *et al.*, 2005;Lomax and Calder, 2008). The prebiotics exert their influence on the immune system both directly by interacting with carbohydrate receptors on immune cells and indirectly by increasing the number of lactic acid producing bacteria. The increase in lactic acid producing bacteria changes the presence of various immunomodulating molecules such as endotoxins, lipopolysaccharides and SCFA (Watzl *et al.*, 2005). This leads to beneficial changes in the immune system such as modulation of immune cells in Peyer´s patches (Hosono *et al.*, 2003;Manhart *et al.*, 2003), increased cytotoxicity of natural killer cells (Kelly-Quagliana *et al.*, 2003) and increased production of IgA and various interleukins (Hosono *et al.*, 2003).

Prebiotics and infection studies in animals

The majority of animal and human studies investigating the effects of prebiotics on susceptibility to infection have been carried out using inulin and fructooligosaccharides (FOS) (Lomax and Calder, 2008).

Most animal studies have been conducted on mice, rats and pigs and the effect of prebiotics have been investigated on a number of pathogenic bacteria including *Escherichia coli* (Bunce *et al.*, 1995) *Clostridium difficile* (Wolf *et al.*, 1997), *Salmonella* typhimurium (Letellier *et al.*, 2000;Buddington *et al.*, 2002;Correa-Matos *et al.*, 2003;Petersen *et al.*, 2009), *Salmonella enteritidis* (Ten Bruggencate *et al.*, 2003;Bovee-Oudenhoven *et al.*, 2003;Ten Bruggencate *et al.*, 2004) and *L. monocytogenes* (Buddington *et al.*, 2002). These studies generally provide evidence to support the hypothesis that prebiotics improve host resistance to bacterial infections. This emerges as prevention of diarrhea, decreased colonization and translocation of the pathogen and increasing animal survival rates.

However, a series of publications by a single group of investigators have investigated the effect of FOS and inulin on infection with *S. enteritidis* in rats on a low-calcium diet (Ten Bruggencate *et al.*, 2003;Bovee-Oudenhoven *et al.*, 2003;Ten Bruggencate *et al.*, 2004;Ten Bruggencate *et al.*, 2005). Despite an increase in the number of faecal lactobacilli and bifidobacteria, increased colonization and translocation of *S. enteritidis* and increased mucosal irritation was seen in these studies. Acidification of the intestine has been shown to be counteracted by dietary calcium (Govers *et al.* 1993). The adverse effect in these studies could be reversed by oral administration of calcium and it was therefore suggested that the increased translocation of *S. enteritidis* could be connected to low pH (Bovee-Oudenhoven *et al.*, 1997;Ten Bruggencate *et al.*, 2004). Roberfoid gives a thorough discussion of these experiments and conclude that they do not demonstrate that FOS and inulin stimulate translocation in rats on a normal diet (Roberfroid, 2005a). A recent study by a different group showed increased translocation of *S.* typhimurium in mouse fed a normal-calcium diet supplemented with FOS (Petersen *et al.*, 2009). However, in this study no decrease in intestinal pH was observed, contradicting that a drop in pH suggested by ten Bruggencate *et al* and Bovee-Oudenhoven *et al* should mediate the increased translocation of Salmonella.

Only one previous study have addressed the effect of prebiotics on the infectivity of *L. monocytogenes* (Buddington *et al.*, 2002). After 6 weeks on a diet supplemented with either

inulin or FOS, mice were given a systemic infection with *L. monocytogenes* by intraperitoneal injection. This way of infecting the animals bypasses the gastrointestinal tract and by that the common starting site of *L. monocytogenes* infections. Nevertheless, feeding with inulin or FOS induced a significant decrease in mortality and it was speculated that the mechanistic basis for the increased resistance could be due to a stimulation of the immune response caused by changes in the intestinal bacterial composition (Buddington *et al*., 2002).

Prebiotics and infection studies in humans

The ability of prebiotics to protect against infection with pathogenic bacteria is more difficult to study in human subjects than in animals since challenge studies are of course not ethically allowed. However, some studies both in children and adults have been conducted up to now. Studies in infants and children have shown a decrease in the incidence of common childhood diarrhoea after prebiotic supplementation (Waligora-Dupriet *et al.*, 2007;Bruzzese *et al.*, 2009). Other studies have failed to show an effect of prebiotics on frequency of common childhood diarrhoea but have instead seen a decrease in the duration or severity of the infections (Saavedra *et al.*, 1999;Tschernia *et al.*, 1999;Juffrie, 2002). Taken together, data from these studies suggest that prebiotics can be used in the prevention and treatment of some infections in infants and children.

Studies in adults are more limited and less convincing compared with the studies made in infants and children. A few studies have investigated the effect of prebiotics on reducing the incidence of travellers´ diarrhoea (Cummings *et al.*, 2001;Drakoularakou *et al.*, 2010). Drakoularakou *et al*., (2010) observed a significantly lower number of episodes with diarrhoea when the subjects consumed GOS prior to and during their holiday. Nevertheless, a study of Cummings *et al*., (2001) showed no effect of FOS supplementation on the incidence of travellers´ diarrhoea. A study including in-patients with *C. difficile*-associated diarrhoea showed a decrease in relapse rates when patients received a diet supplemented with FOS (Lewis *et al.*, 2005a). However, a study where elderly patients were given a diet supplemented with FOS showed no protection against antibiotic-associated diarrhoea, whether caused by *C. difficile* or other bacteria (Lewis *et al.*, 2005b).

Although more human studies needs to be done before any clear conclusion can be drawn, evidence from both animal and human studies accumulate to suggest that consumption of some prebiotics can reduce the incidence or duration of some infections in children and adults.

Discussion

This study presents an investigation of the effects of different non-digestible carbohydrates (putative prebiotics) on the infective potential of *L. monocytogenes.*

It was found that feeding guinea pigs with inulin and apple pectin promoted *L. monocytogenes* infection, while xylooligosaccharide (XOS) and galactooligosaccharide (GOS) prevented it. No effect was seen after feeding the guinea pigs with polydextrose (manuscript I).

Several studies have suggested that short chain carbohydrates are fermented more rapidly with greater bifidogenic effect than carbohydrates with longer chain length (DP>10) (Livesey *et al.*, 1993;de, V *et al.*, 2008;Hernot *et al.*, 2009). XOS and GOS both have a degree of polymerization between 2 and 6 while the three other carbohydrates tested all have a greater degree of polymerization. It is therefore striking that XOS and GOS also are the two carbohydrates preventing *L. monocytogenes* infection in guinea pigs. Because of the short chain length it could therefore be speculated that the fermentation of XOS and GOS by the microbiota already starts in the small intestine. Many gut infections, including infection with *L. monocytogenes*, occur in the small intestine. However, the microbiota-dependent effect of prebiotics is generally believed to be limited in the small intestine since the fermentation of prebiotics mainly occurs in the colon where the bacteria density is highest. However, if the fermentation of XOS and GOS, because of their low DP, already starts in the small intestine the number of beneficial bacteria might increase explaining why it is only XOS and GOS that have been found to prevent infections with *L. monocytogenes* in guinea pigs. XOS and GOS might also in themselves or through stimulation of lactobacilli and bifidobacteria have a larger impact on the immune system than the other three carbohydrates. This could be a systemic stimulation of the immune system, independent of the site of prebiotic fermentation.

In this study XOS was found to have a protective effect against infections with *L. monocytogenes*, however it has recently been demonstrated that XOS increased the translocation of *Salmonella* Typhimurium in mice (Petersen *et al.*, 2009). This indicates that the prebiotic effect is dependent on the pathogenic species tested and probably also on the animal model used.

Feeding the guinea pigs with XOS and polydextrose led to a drop in caecal pH. This indicates a change in the fermentation pattern and a change in the composition of the microbiota. Faecal samples were obtained from the guinea pigs before and after feeding with prebiotics. Future

examination of the composition of the guinea pig microbiota in these samples would give us information about what effect the carbohydrates have on the composition of the microbiota. This could possibly help explain some of the observed *in vivo* effects seen in the infection assay. Relatively little is known about the microbiota of guinea pigs. Examination of the microbiota of guinea pigs will give us the opportunity to compare with the microbiota of humans. This might strengthen our ability to interpret the results from the guinea pig infection study and to predict what impact the prebiotics will have on enteric infections in humans.

So far, only one study has investigated the effect of prebiotics on the infective potential of *L. monocytogenes* (Buddington *et al.*, 2002). Contradictory to my results, Buddington *et al*. reported that feeding with inulin substantially reduced the mortality rate of mice after infection with *L. monocytogenes*. However, the setup of the two experiments is quite different and the results are therefore difficult to compare. Different *L. monocytogenes* strains, different animal models, different way of infecting the animals and different ways of measuring the infectivity of *L. monocytogenes* were used in the two studies. All factors that potentially could have an impact on the outcome of the experiments.

It has up to now been believed that InlB was not involved in the intestinal phase of the listerial infection. In this study we primary wanted to investigate the intestinal phase of *L. monocytogenes* infection and guinea pigs compared with mice were therefore the obvious choice as animal model. However, a recent study suggests that InlB might be involved in the intestinal phase of the *L. monocytogenes* infection (Pentecost *et al*., 2010). More studies are needed to further elucidate which role InlB plays in the gastrointestinal colonization and invasion of *L. monocytogenes*. This might provide a background for an evaluation of the relevance in using guinea pigs in future studies of *L. monocytogenes*.

The attention has recently been drawn to the gerbil as an animal model for listeriosis (Disson *et al.*, 2008). The gerbil is naturally susceptible to *L. monocytogenes* and was one of the first animals from where *L. monocytogenes* was isolated (Pirie, 1927). The biology of gerbils is not thoroughly elucidated, but according to Disson *et al* the gerbil is easy to work with and give robust results (Disson *et al.*, 2008;Disson *et al.*, 2009). More infection studies with the gerbil are needed but it has, together with the knock-in E16P mouse line, potential to be the future choice of animal model for infection studies with *L. monocytogenes*.

The guinea pigs were in this present study given a diet supplemented with 10% of the prebiotics. Most animal infection studies use between 6-16% of prebiotics in the diet (Bubbington *et al*., 2002;Ten Buggencate *et al*., 2004;Petkevicius *et al*., 2007;Petersen *et al*., 2009). This greatly exceeds the maximum dose of prebiotics of 20 g/day that is generally recognized as safe for humans (Douglas and Sanders, 2008). However, this high dose is used to reduce the risk of missing a prebiotic effect because the dose is too small. If a positive prebiotic effect is observed randomized clinical trials have to be conducted with a smaller amount of prebiotic to confirm beneficial effects in humans and to demonstrate the absence of harmful effects.

To further explore the mechanisms behind the effects observed *in vivo* the microbiota-independent effects of the same four indigestible carbohydrates were investigated (manuscript II). It should be noted that apple pectin was not included in the adherence assay and the qPCR analyses since it formed a gel-structure when it was dissolved in water.

When XOS was added immediately prior to the start of the adherence assay the adherence of strain 7291 and ScottA, but not of strain 4446 to tissue culture cells was inhibited. As the only strain of the three *L. monocytogenes* strains, 4446 is capable of fermenting XOS. It could be speculated that strain 4446 rapidly takes up XOS and in that way prevents it from sticking to the cell surface blocking adherence to the intestinal cells. These results suggest that XOS might act as a receptor decoy for *L. monocytogenes* strain 7291 and ScottA preventing attachment to the intestinal enterocytes. This might help explain the decrease in the severity of *L. monocytogenes* infection seen in guinea pigs fed with XOS. GOS also prevented infection with *L. monocytogenes in vivo*, however, no effect of this carbohydrate was seen on the *in vitro* adherence of *L. monocytogenes* to intestinal cells. Different mechanisms may therefore be responsible for the preventive effect of XOS and GOS seen in guinea pigs.

It has previously been shown that prebiotics not only are capable of preventing *in vitro* adherence of pathogens but also of increasing the adherence (Searle *et al.*, 2009). However, despite the increased infectivity after inulin feeding, inulin had no effect on the adherence of *L. monocytogenes in vitro*. Polydextrose had no effect *in vivo* or *in vitro*.

The theory behind the anti-adherence effect of some prebiotics is based on the observation that many pathogens utilize saccharide-containing glycoproteins as their cellular adhesion receptor on the surface of intestinal cells. The prebiotics might work as a receptor decoy for the bacteria

preventing it from binding to the intestinal cells. This may very well be correct, however, to my knowledge no studies have so far investigated the possibility that the prebiotic instead of binding to the bacteria binds to the intestinal cell and in that way blocks the adherence of the bacteria. To investigate this, the intestinal cells could be pre-incubated with the prebiotic followed by a thorough washing before adding the bacteria.

qPCR was used to investigate if incubation of *L. monocytogenes* with the carbohydrates could affect expression of genes known to be involved in the intestinal adherence and invasion of *L. monocytogenes*. The expression profile of strain 7291 was highly different from the expression profile of strain 4446 and ScottA that were very similar. The four prebiotics had an effect on most genes of strain 4446 and ScottA while the only gene significantly affected in strain 7291 was *iap*. The adherence of all three strains of *L. monocytogenes* was reduced after two hours of incubation with XOS and subsequently removal of the carbohydrate, thus the unique expression profile of 7291 was not reflected in the *in vitro* adherence assay. Strain 7291 also separated itself from strain 4446 and ScottA in other ways. In the infection assay strain 7291 was slightly more predominant in the intestine and organs than strain 4446 and ScottA. However, feeding the guinea pigs with the carbohydrates did not result in a different distribution in the intestine or relative translocation of the three strains compared with the control groups. Furthermore, in the fermentation assay strain 4446 was able to utilize all the five tested carbohydrates, ScottA could utilize all except XOS and polydextrose while strain 7291 only was able to utilize GOS. It could therefore be speculated that the different expression profile of strain 7291 might somehow be explained by the lack of ability to ferment most of the carbohydrates. It should be noted that the growth OD was comparable for the three strains after two hours of incubation with the carbohydrates. This indicates that all of the three strains still used the glucose present in the media as growth substrate.

The expression of *inlA* of strain 4446 and ScottA were down regulated by all of the four prebiotics. However, XOS had the strongest effect on *inlA* expression. This correlates with data from the adherence assay showing that only XOS reduces the adherence of *L. monocytogenes* to the intestinal tissue culture cells. InlA is known to be the principal protein involved in the adherence of *L. monocytogenes* to intestinal cells. It has previously been shown that a correlation exists between the level of *inlA* expression and the invasion capacity into Caco-2 cells (Werbrouck *et al.*, 2006). It could be speculated that the expression of *inlA* has to be reduced

below a certain level before it results in reduction of the adherence in the *in vitro* adherence assay.

Also other adherence genes were affected by the presence of the carbohydrates. It is very likely interactions between different proteins that are responsible for the ability of *L. monocytogenes* to adhere to the intestinal cells. However, the exact role and interplay of all the different proteins observed to be involved in adherence of *L. monocytogenes* to intestinal cells is not yet elucidated. Nonetheless, incubation with the carbohydrates changed the expression of most of the tested genes of strain 4446 and ScottA and this change in expression might be one of the explanations of the prebiotics effects seen *in vivo.*

However, these experiments were done *in vitro* and nothing is known about what impact these carbohydrates will have *in vivo* on expression of adherence genes in *L. monocytogenes*. It could therefore be interesting to examine the expression of *L. monocytogenes* adherence genes in intestinal samples taken from guinea pigs fed with prebiotics. Nevertheless, because of quick degradation of mRNA it might be difficult to investigate the gene expression *in vivo*. However, instead of looking at the gene expression it might be possible e.g. by Western blotting to look at the protein expression *in vivo*.

Conclusion

Experiments in the present study show that some carbohydrates promote and some carbohydrates protect against infection with *L. monocytogenes* in guinea pigs. The data furthermore suggest that dietary carbohydrates may alter the infective potential of *L. monocytogenes* by affecting the expression of adhesion-related genes, as well as through directly sticking to the bacterial surface. This means that certain dietary carbohydrates may prevent *L. monocytogenes* infections through other mechanisms than merely a beneficial effect on the gut microbiota.

The carbohydrates probably affect several mechanisms that all in their way influence the infective potential of *L. monocytogenes*. It is the sum of these changes that result in the effect seen in the guinea pig infection assay. Results from this present study furthermore suggest that the mechanisms implicated are very dependent on the strain and carbohydrate involved.

This study supports the hypothesis that some prebiotics can be helpful in the prevention of intestinal infections. The dietary use of prebiotics holds much potential, however, this study also shows that it is not safe to automatically assume that prebiotics only have positive or no effects on the infectivity of pathogens in the intestine.

More knowledge about the underlying mechanisms responsible for the positive and negative effects of prebiotics on the infective potential of pathogens might lead to selection and development of prebiotics with increased effect against enteric pathogenic bacteria.

Reference List

Adlerberth,I. (2008). Factors influencing the establishment of the intestinal microbiota in infancy. Nestle.Nutr.Workshop Ser.Pediatr.Program. *62*, 13-29.

Akpinar,O., Erdogan,K., Bakir,U., and Yilmaz,L. (2010). Comparison of acid and enzymatic hydrolysis of tobacco stalk xylan for preparation of xylooligosaccharides. Lwt-Food Science and Technology *43*, 119-125.

Akpinar,O., Erdogan,K., and Bostanci,S. (2009). Production of xylooligosaccharides by controlled acid hydrolysis of lignocellulosic materials. Carbohydrate Research *344*, 660-666.

Albrecht,S., Schols,H.A., Klarenbeek,B., Voragen,A.G.J., and Gruppen,H. (2010). Introducing Capillary Electrophoresis with Laser-Induced Fluorescence (CE-LIF) as a Potential Analysis and Quantification Tool for Galactooligosaccharides Extracted from Complex Food Matrices. Journal of Agricultural and Food Chemistry *58*, 2787-2794.

Altenhoefer,A., Oswald,S., Sonnenborn,U., Enders,C., Schulze,J., Hacker,J., and Oelschlaeger,T.A. (2004). The probiotic Escherichia coli strain Nissle 1917 interferes with invasion of human intestinal epithelial cells by different enteroinvasive bacterial pathogens. Fems Immunology and Medical Microbiology *40*, 223-229.

Andersen,J.B., Roldgaard,B.B., Christensen,B.B., and Licht,T.R. (2007). Oxygen restriction increases the infective potential of Listeria monocytogenes in vitro in Caco-2 cells and in vivo in guinea pigs. BioMed Central Microbiology *7*, 55.

Ankenbauer,R.G. and Nester,E.W. (1990). Sugar-Mediated Induction of Agrobacterium-Tumefaciens Virulence Genes - Structural Specificity and Activities of Monosaccharides. Journal of Bacteriology *172*, 6442-6446.

Bambirra,F.H., Lima,K.G., Franco,B.D., Cara,D.C., Nardi,R.M., Barbosa,F.H., and Nicoli,J.R. (2007). Protective effect of Lactobacillus sakei 2a against experimental challenge with Listeria monocytogenes in gnotobiotic mice. Lett.Appl.Microbiol. *45*, 663-667.

Barcelo,A., Claustre,J., Moro,F., Chayvialle,J.A., Cuber,J.C., and Plaisancie,P. (2000). Mucin secretion is modulated by luminal factors in the isolated vascularly perfused rat colon. Gut *46*, 218-224.

Bartosch,S., Fite,A., Macfarlane,G.T., and McMurdo,M.E. (2004). Characterization of bacterial communities in feces from healthy elderly volunteers and hospitalized elderly patients by using real-time PCR and effects of antibiotic treatment on the fecal microbiota. Appl.Environ.Microbiol. *70*, 3575-3581.

Begley,M., Gahan,C.G., and Hill,C. (2005). The interaction between bacteria and bile. FEMS Microbiol.Rev. *29*, 625-651.

Bik,E.M. (2009). Composition and function of the human-associated microbiota. Nutr.Rev. *67 Suppl 2*, S164-S171.

Boehm,G., Lidestri,M., Casetta,P., Jelinek,J., Negretti,F., Stahl,B., and Marini,A. (2002). Supplementation of a bovine milk formula with an oligosaccharide mixture increases counts of faecal bifidobacteria in preterm infants. Arch.Dis.Child Fetal Neonatal Ed *86*, F178-F181.

Boesten,R.J. and de Vos,W.M. (2008). Interactomics in the human intestine: Lactobacilli and Bifidobacteria make a difference. J.Clin.Gastroenterol. *42 Suppl 3 Pt 2*, S163-S167.

Bonazzi,M., Lecuit,M., and Cossart,P. (2009). Listeria monocytogenes internalin and E-cadherin: from structure to pathogenesis. Cell Microbiol.

Bosscher,D., Loo,J.V., and Franck,A. (2006). Inulin and oligofructose as prebiotics in the prevention of intestinal infections and diseases. Nutr.Res.Rev. *19*, 216-226.

Bouhnik,Y., Flourie,B., gay-Abensour,L., Pochart,P., Gramet,G., Durand,M., and Rambaud,J.C. (1997). Administration of transgalacto-oligosaccharides increases fecal bifidobacteria and modifies colonic fermentation metabolism in healthy humans. J.Nutr. *127*, 444-448.

Bouhnik,Y., Flourie,B., Riottot,M., Bisetti,N., Gailing,M.F., Guibert,A., Bornet,F., and Rambaud,J.C. (1996). Effects of fructo-oligosaccharides ingestion on fecal bifidobacteria and selected metabolic indexes of colon carcinogenesis in healthy humans. Nutr.Cancer *26*, 21-29.

Bourlioux,P., Koletzko,B., Guarner,F., and Braesco,V. (2003). The intestine and its microflora are partners for the protection of the host: report on the Danone Symposium "The Intelligent Intestine," held in Paris, June 14, 2002. Am.J.Clin.Nutr. *78*, 675-683.

Bovee-Oudenhoven,I.M., Termont,D.S., Heidt,P.J., and Van der,M.R. (1997). Increasing the intestinal resistance of rats to the invasive pathogen Salmonella enteritidis: additive effects of dietary lactulose and calcium. Gut *40*, 497-504.

Bovee-Oudenhoven,I.M.J., Ten Bruggencate,S.J.M., Lettink-Wissink,M.L.G., and Van der Meer,R. (2003). Dietary fructo-oligosaccharides and lactulose inhibit intestinal colonisation but stimulate translocation of salmonella in rats. Gut *52*, 1572-1578.

Boyle,R.J. and Tang,M.L. (2006). The role of probiotics in the management of allergic disease. Clin.Exp.Allergy *36*, 568-576.

Braun,L., Dramsi,S., Dehoux,P., Bierne,H., Lindahl,G., and Cossart,P. (1997). InlB: an invasion protein of Listeria monocytogenes with a novel type of surface association. Mol.Microbiol. *25*, 285-294.

Braun,L., Ghebrehiwet,B., and Cossart,P. (2000). gC1q-R/p32, a C1q-binding protein, is a receptor for the InlB invasion protein of Listeria monocytogenes. EMBO J. *19*, 1458-1466.

Breazile,J.E. and Brown,E.M. (1976). Anatomy. In Joseph E Wagner and Patrick J Manning (Ed.) The Biology of the Guinea Pig. Academic Press, Inc, New York, 53-62.

Brencic,A. and Winans,S.C. (2005). Detection of and response to signals involved in host-microbe interactions by plant-associated bacteria. Microbiology and Molecular Biology Reviews *69*, 155-+.

Bruzzese,E., Volpicelli,M., Squaglia,M., Tartaglione,A., and Guarino,A. (2006). Impact of prebiotics on human health. Dig.Liver Dis. *38 Suppl 2*, S283-S287.

Bruzzese,E. *et al.* (2009). A formula containing galacto- and fructo-oligosaccharides prevents intestinal and extra-intestinal infections: an observational study. Clin.Nutr. *28*, 156-161.

Bublitz,M., Polle,L., Holland,C., Heinz,D.W., Nimtz,M., and Schubert,W.D. (2009). Structural basis for autoinhibition and activation of Auto, a virulence-associated peptidoglycan hydrolase of Listeria monocytogenes. Mol.Microbiol. *71*, 1509-1522.

Buddington,K.K., Donahoo,J.B., and Buddington,R.K. (2002). Dietary oligofructose and inulin protect mice from enteric and systemic pathogens and tumor inducers. J.Nutr. *132*, 472-477.

Bunce,T., Howard,M.D., Kerley,M.S., Allee,G.L., and Pace,L.W. (1995). Protective effect of frucooligosaccharides (FOS) in prevention of mortality and morbidity from infectious E. coli. Journal of Animal Science *71*, 69.

Burdock,G.A. and Flamm,W.G. (1999). A review of the studies of the safety of polydextrose in food. Food and Chemical Toxicology *37*, 233-264.

Burkholder,K.M., Kim,K.P., Mishra,K.K., Medina,S., Hahm,B.K., Kim,H., and Bhunia,A.K. (2009). Expression of LAP, a SecA2-dependent secretory protein, is induced under anaerobic environment. Microbes.Infect. *11*, 859-867.

Cabanes,D., Dussurget,O., Dehoux,P., and Cossart,P. (2004). Auto, a surface associated autolysin of Listeria monocytogenes required for entry into eukaryotic cells and virulence. Mol.Microbiol. *51*, 1601-1614.

Caffall,K.H. and Mohnen,D. (2009). The structure, function, and biosynthesis of plant cell wall pectic polysaccharides. Carbohydrate Research *344*, 1879-1900.

Campbell,J.M., Fahey,G.C., Jr., and Wolf,B.W. (1997). Selected indigestible oligosaccharides affect large bowel mass, cecal and fecal short-chain fatty acids, pH and microflora in rats. J.Nutr. *127*, 130-136.

Casadei,G., Grilli,E., and Piva,A. (2009). Pediocin A modulates intestinal microflora metabolism in swine in vitro intestinal fermentations. Journal of Animal Science *87*, 2020-2028.

Chen,Y.S., Srionnual,S., Onda,T., and Yanagida,F. (2007). Effects of prebiotic oligosaccharides and trehalose on growth and production of bacteriocins by lactic acid bacteria. Letters in Applied Microbiology *45*, 190-193.

Collado,M.C., Gueimonde,M., Hernandez,M., Sanz,Y., and Salminen,S. (2005). Adhesion of selected Bifidobacterium strains to human intestinal mucus and the role of adhesion in enteropathogen exclusion. J.Food Prot. *68*, 2672-2678.

Collado,M.C., Jalonen,L., Meriluoto,J., and Salminen,S. (2006). Protection mechanism of probiotic combination against human pathogens: in vitro adhesion to human intestinal mucus. Asia Pac.J.Clin.Nutr. *15*, 570-575.

Corr,S., Hill,C., and Gahan,C.G. (2006). An in vitro cell-culture model demonstrates internalin- and hemolysin-independent translocation of Listeria monocytogenes across M cells. Microb.Pathog. *41*, 241-250.

Corr,S.C., Gahan,C.G.M., and Hill,C. (2007). Impact of selected Lactobacillus and Bifidobacterium species on Listeria monocytogenes infection and the mucosal immune response. Fems Immunology and Medical Microbiology *50*, 380-388.

Correa-Matos,N.J., Donovan,S.M., Isaacson,R.E., Gaskins,H.R., White,B.A., and Tappenden,K.A. (2003). Fermentable fiber reduces recovery time and improves intestinal function in piglets following Salmonella typhimurium infection. J.Nutr. *133*, 1845-1852.

Courtney,H.S., Li,Y., Dale,J.B., and Hasty,D.L. (1994). Cloning, sequencing, and expression of a fibronectin/fibrinogen-binding protein from group A streptococci. Infect.Immun. *62*, 3937-3946.

Crociani,F., Allesandrini,A., Mucci,M.M.B., and Biavati,B. (1994). Degradation of Complex Carbohydrates by Bifidobacterium Spp. International Journal of Food Microbiology *24*, 199-210.

Cummings,J.H., Christie,S., and Cole,T.J. (2001). A study of fructo oligosaccharides in the prevention of travellers' diarrhoea. Aliment.Pharmacol.Ther. *15*, 1139-1145.

Cummings,J.H. and Englyst,H.N. (1987). Fermentation in the Human Large-Intestine and the Available Substrates. American Journal of Clinical Nutrition *45*, 1243-1255.

Cummings,J.H. and Macfarlane,G.T. (1991). The Control and Consequences of Bacterial Fermentation in the Human Colon. Journal of Applied Bacteriology *70*, 443-459.

Cummins,A.J., Fielding,A.K., and McLauchlin,J. (1994). Listeria ivanovii infection in a patient with AIDS. J.Infect. *28*, 89-91.

Daniels,J.J., Autenrieth,I.B., and Goebel,W. (2000). Interaction of Listeria monocytogenes with the intestinal epithelium. FEMS Microbiol.Lett. *190*, 323-328.

Davis,M.J., Coote,P.J., and O'Byrne,C.P. (1996). Acid tolerance in Listeria monocytogenes: the adaptive acid tolerance response (ATR) and growth-phase-dependent acid resistance. Microbiology *142 (Pt 10)*, 2975-2982.

de,P., V, Vanhoutte,T., Huys,G., Swings,J., Rutgeerts,P., and Verbeke,K. (2008). Baseline microbiota activity and initial bifidobacteria counts influence responses to prebiotic dosing in healthy subjects. Aliment.Pharmacol.Ther. *27*, 504-513.

Despopoulos,A. and Silbernagl,S. (1991). Nutrition and Digestion In Color Atlas of Physiology by Wolf-Rüdiger Gay and Astried Rothenburger (Ed.). Thieme Medical Publishers, Inc. New York, 196-231.

Dethlefsen,L., Fall-Ngai,M., and Relman,D.A. (2007). An ecological and evolutionary perspective on human-microbe mutualism and disease. Nature *449*, 811-818.

Disson,O. *et al.* (2008). Conjugated action of two species-specific invasion proteins for fetoplacental listeriosis. Nature *455*, 1114-1118.

Disson,O., Nikitas,G., Grayo,S., Dussurget,O., Cossart,P., and Lecuit,M. (2009). Modeling human listeriosis in natural and genetically engineered animals. Nat.Protoc. *4*, 799-810.

Donnelly,C.W. (2001). Listeria monocytogenes: a continuing challenge. Nutr.Rev. *59*, 183-194.

Douglas,L.C. and Sanders,M.E. (2008). Probiotics and prebiotics in dietetics practice. Journal of the American Dietetic Association. *108*, 510-521.

Drakoularakou,A., Tzortzis,G., Rastall,R.A., and Gibson,G.R. (2010). A double-blind, placebo-controlled, randomized human study assessing the capacity of a novel galacto-oligosaccharide mixture in reducing travellers' diarrhoea. European Journal of Clinical Nutrition *64*, 146-152.

Dramsi,S., Bourdichon,F., Cabanes,D., Lecuit,M., Fsihi,H., and Cossart,P. (2004). FbpA, a novel multifunctional Listeria monocytogenes virulence factor. Mol.Microbiol. *53*, 639-649.

Dussurget,O., Cabanes,D., Dehoux,P., Lecuit,M., Buchrieser,C., Glaser,P., and Cossart,P. (2002). Listeria monocytogenes bile salt hydrolase is a PrfA-regulated virulence factor involved in the intestinal and hepatic phases of listeriosis. Mol.Microbiol. *45*, 1095-1106.

Eckburg,P.B., Bik,E.M., Bernstein,C.N., Purdom,E., Dethlefsen,L., Sargent,M., Gill,S.R., Nelson,K.E., and Relman,D.A. (2005). Diversity of the human intestinal microbial flora. Science *308*, 1635-1638.

EFSA Annual Report (2007). The Community Summary Report in Trends and Sources of Zoonoses and Zoonotic Agents in the European Union in 2007.

Erichsen,S. (1969). Marsvinfamilien - Caviidae. In: Forsøksdyrbiologi Universitetsforlaget, Oslo, 38-42.

Faith,N.G., Kathariou,S., Neudeck,B.L., Luchansky,J.B., and Czuprynski,C.J. (2007). A P60 mutant of Listeria monocytogenes is impaired in its ability to cause infection in intragastrically inoculated mice. Microb.Pathog. *42*, 237-241.

Fava,F., Makivuokko,H., Siljander-Rasi,H., Putaala,H., Tiihonen,K., Stowell,J., Tuohy,K., Gibson,G., and Rautonen,N. (2007). Effect of polydextrose on intestinal microbes and immune functions in pigs. British Journal of Nutrition *98*, 123-133.

Ferreira,M.A. and Lund,B.M. (1996). The effect of nisin on Listeria monocytogenes in culture medium and long-life cottage cheese. Lett.Appl.Microbiol. *22*, 433-438.

Flint,H.J., Duncan,S.H., Scott,K.P., and Louis,P. (2007). Interactions and competition within the microbial community of the human colon: links between diet and health. Environ.Microbiol. *9*, 1101-1111.

Fooks,L.J. and Gibson,G.R. (2002). In vitro investigations of the effect of probiotics and prebiotics on selected human intestinal pathogens. FEMS Microbiol.Ecol. *39*, 67-75.

Fowler,T., Wann,E.R., Joh,D., Johansson,S., Foster,T.J., and Hook,M. (2000). Cellular invasion by Staphylococcus aureus involves a fibronectin bridge between the bacterial fibronectin-binding MSCRAMMs and host cell beta1 integrins. Eur.J.Cell Biol. *79*, 672-679.

Fukasawa,T. *et al.* (2007). Identification of marker genes for intestinal immunomodulating effect of a fructooligosaccharide by DNA microarray analysis. Journal of Agricultural and Food Chemistry *55*, 3174-3179.

Gahan,C.G. and Hill,C. (2005). Gastrointestinal phase of Listeria monocytogenes infection. J.Appl.Microbiol. *98*, 1345-1353.

Gaillard,J.L., Berche,P., Frehel,C., Gouin,E., and Cossart,P. (1991). Entry of L. monocytogenes into cells is mediated by internalin, a repeat protein reminiscent of surface antigens from gram-positive cocci. Cell *65*, 1127-1141.

Galvez,A., Lopez,R.L., Abriouel,H., Valdivia,E., and Ben Omar,N. (2008). Application of bacteriocins in the control of foodborne pathogenic and spoilage bacteria. Critical Reviews in Biotechnology *28*, 125-152.

Gibson,G.R. and Fuller,R. (2000). Aspects of in vitro and in vivo research approaches directed toward identifying probiotics and prebiotics for human use. J.Nutr. *130*, 391S-395S.

Gibson,G.R., Probert,H.M., Loo,J.V., Rastall,R.A., and Roberfroid,M.B. (2004). Dietary modulation of the human colonic microbiota: updating the concept of prebiotics. Nutr.Res.Rev. *17*, 259-275.

Gibson,G.R. and Wang,X. (1994). Enrichment of bifidobacteria from human gut contents by oligofructose using continuous culture. FEMS Microbiol.Lett. *118*, 121-127.

Gilbreth,S.E., Benson,A.K., and Hutkins,R.W. (2004). Catabolite repression and virulence gene expression in Listeria monocytogenes. Curr.Microbiol. *49*, 95-98.

Gilot,P., Andre,P., and Content,J. (1999). Listeria monocytogenes possesses adhesins for fibronectin. Infect.Immun. *67*, 6698-6701.

Gilot,P., Jossin,Y., and Content,J. (2000). Cloning, sequencing and characterisation of a Listeria monocytogenes gene encoding a fibronectin-binding protein. J.Med.Microbiol. *49*, 887-896.

Glaser,P. *et al.* (2001). Comparative genomics of Listeria species. Science *294*, 849-852.

Gray,M.L. and Killinger,A.H. (1966). Listeria monocytogenes and listeric infections. Bacteriol.Rev. *30*, 309-382.

Guarner,F. and Malagelada,J.R. (2003). Gut flora in health and disease. Lancet *361*, 512-519.

Guillet,C. *et al.* (2010). Human listeriosis caused by Listeria ivanovii. Emerg.Infect.Dis. *16*, 136-138.

Hamon,M., Bierne,H., and Cossart,P. (2006). Listeria monocytogenes: a multifaceted model. Nat.Rev.Microbiol. *4*, 423-434.

Hardy,J., Francis,K.P., DeBoer,M., Chu,P., Gibbs,K., and Contag,C.H. (2004). Extracellular replication of Listeria monocytogenes in the murine gall bladder. Science *303*, 851-853.

Hengst,C., Ptok,S., Roessler,A., Fechner,A., and Jahreis,G. (2009). Effects of polydextrose supplementation on different faecal parameters in healthy volunteers. International Journal of Food Sciences and Nutrition *60*, 96-105.

Hernot,D.C., Boileau,T.W., Bauer,L.L., Middelbos,I.S., Murphy,M.R., Swanson,K.S., and Fahey,G.C., Jr. (2009). In vitro fermentation profiles, gas production rates, and microbiota modulation as affected by certain fructans, galactooligosaccharides, and polydextrose. J.Agric.Food Chem. *57*, 1354-1361.

Holzapfel,W.H., Haberer,P., Snel,J., Schillinger,U., and Huis,i., V (1998). Overview of gut flora and probiotics. Int.J.Food Microbiol. *41*, 85-101.

Hosono,A., Ozawa,A., Kato,R., Ohnishi,Y., Nakanishi,Y., Kimura,T., and Nakamura,R. (2003). Dietary fructooligosaccharides induce immunoregulation of intestinal IgA secretion by murine Peyer's patch cells. Bioscience Biotechnology and Biochemistry *67*, 758-764.

Ito,M., Kimura,M., Deguchi,Y., Miyamori-Watabe,A., Yajima,T., and Kan,T. (1993). Effects of transgalactosylated disaccharides on the human intestinal microflora and their metabolism. J.Nutr.Sci.Vitaminol.(Tokyo) *39*, 279-288.

Jacquet,C., Doumith,M., Gordon,J.I., Martin,P.M., Cossart,P., and Lecuit,M. (2004). A molecular marker for evaluating the pathogenic potential of foodborne Listeria monocytogenes. J.Infect.Dis. *189*, 2094-2100.

Janer,C., Rohr,L.M., Pelaez,C., Laloi,M., Cleusix,V., Requena,T., and Meile,L. (2004). Hydrolysis of oligofructoses by the recombinant beta-fructofuranosidase from Bifidobacterium lactis. Syst.Appl.Microbiol. *27*, 279-285.

Jaradat,Z.W. and Bhunia,A.K. (2002). Glucose and nutrient concentrations affect the expression of a 104-kilodalton Listeria adhesion protein in Listeria monocytogenes. Appl.Environ.Microbiol. *68*, 4876-4883.

Jaradat,Z.W., Wampler,J.W., and Bhunia,A.W. (2003). A Listeria adhesion protein-deficient Listeria monocytogenes strain shows reduced adhesion primarily to intestinal cell lines. Med.Microbiol.Immunol. *192*, 85-91.

Jaskari,J., Kontula,P., Siitonen,A., Jousimies-Somer,H., Mattila-Sandholm,T., and Poutanen,K. (1998). Oat beta-glucan and xylan hydrolysates as selective substrates for Bifidobacterium and Lactobacillus strains. Appl.Microbiol.Biotechnol. *49*, 175-181.

Johansson,J., Mandin,P., Renzoni,A., Chiaruttini,C., Springer,M., and Cossart,P. (2002). An RNA thermosensor controls expression of virulence genes in Listeria monocytogenes. Cell *110*, 551-561.

Jonquieres,R., Bierne,H., Mengaud,J., and Cossart,P. (1998). The inlA gene of Listeria monocytogenes LO28 harbors a nonsense mutation resulting in release of internalin. Infect.Immun. *66*, 3420-3422.

Juffrie,M. (2002). Fructooligosaccharide and diarrhea. Bioscience and Microflora *21*, 31-34.

Kararli,T.T. (1995). Comparison of the gastrointestinal anatomy, physiology, and biochemistry of humans and commonly used laboratory animals. Biopharm.Drug Dispos. *16*, 351-380.

Kelly-Quagliana,K.A., Nelson,P.D., and Buddington,R.K. (2003). Dietary oligofructose and inulin modulate immune functions in mice. Nutrition Research *23*, 257-267.

Khelef,N., Lecuit,M., Bierne,H., and Cossart,P. (2006). Species specificity of the Listeria monocytogenes InlB protein. Cell Microbiol. *8*, 457-470.

Kim,K.P., Jagadeesan,B., Burkholder,K.M., Jaradat,Z.W., Wampler,J.L., Lathrop,A.A., Morgan,M.T., and Bhunia,A.K. (2006). Adhesion characteristics of Listeria adhesion protein (LAP)-expressing

Escherichia coli to Caco-2 cells and of recombinant LAP to eukaryotic receptor Hsp60 as examined in a surface plasmon resonance sensor. FEMS Microbiol.Lett. *256*, 324-332.

Knutton,S., AduBobie,J., Bain,C., Phillips,A.D., Dougan,G., and Frankel,G. (1997). Down regulation of intimin expression during attaching and effacing enteropathogenic Escherichia coli adhesion. Infection and Immunity *65*, 1644-1652.

Kuhn,M. and Goebel,W. (1989). Identification of an extracellular protein of Listeria monocytogenes possibly involved in intracellular uptake by mammalian cells. Infect.Immun. *57*, 55-61.

Kunz,C., Rudloff,S., Baier,W., Klein,N., and Strobel,S. (2000). Oligosaccharides in human milk: structural, functional, and metabolic aspects. Annu.Rev.Nutr. *20*, 699-722.

Kutchai,H.C. (1998). The Gastrointestinal System: In Robert M. Berne Et. Al (Ed.), Physiology. 589-674.

Larsen,M.H., Kallipolitis,B.H., Christiansen,J.K., Olsen,J.E., and Ingmer,H. (2006). The response regulator ResD modulates virulence gene expression in response to carbohydrates in Listeria monocytogenes. Mol.Microbiol. *61*, 1622-1635.

Lecuit,M. (2007). Human listeriosis and animal models. Microbes.Infect. *9*, 1216-1225.

Lecuit,M., Dramsi,S., Gottardi,C., Fedor-Chaiken,M., Gumbiner,B., and Cossart,P. (1999). A single amino acid in E-cadherin responsible for host specificity towards the human pathogen Listeria monocytogenes. EMBO J. *18*, 3956-3963.

Lecuit,M., Ohayon,H., Braun,L., Mengaud,J., and Cossart,P. (1997). Internalin of Listeria monocytogenes with an intact leucine-rich repeat region is sufficient to promote internalization. Infect.Immun. *65*, 5309-5319.

Lecuit,M., Vandormael-Pournin,S., Lefort,J., Huerre,M., Gounon,P., Dupuy,C., Babinet,C., and Cossart,P. (2001). A transgenic model for listeriosis: role of internalin in crossing the intestinal barrier. Science *292*, 1722-1725.

Lee,Y.K., Puong,K.Y., Ouwehand,A.C., and Salminen,S. (2003). Displacement of bacterial pathogens from mucus and Caco-2 cell surface by lactobacilli. J.Med.Microbiol. *52*, 925-930.

Leimeister-Wachter,M., Domann,E., and Chakraborty,T. (1992). The expression of virulence genes in Listeria monocytogenes is thermoregulated. J.Bacteriol. *174*, 947-952.

Leser,T.D. and Molbak,L. (2009). Better living through microbial action: the benefits of the mammalian gastrointestinal microbiota on the host. Environ.Microbiol. *11*, 2194-2206.

Letellier,A., Messier,S., Lessard,L., and Quessy,S. (2000). Assessment of various treatments to reduce carriage of Salmonella in swine. Can.J.Vet.Res. *64*, 27-31.

Lewis,S., Burmeister,S., and Brazier,J. (2005a). Effect of the prebiotic oligofructose on relapse of Clostridium difficile-associated diarrhea: a randomized, controlled study. Clin.Gastroenterol.Hepatol. *3*, 442-448.

Lewis,S., Burmeister,S., Cohen,S., Brazier,J., and Awasthi,A. (2005b). Failure of dietary oligofructose to prevent antibiotic-associated diarrhoea. Aliment.Pharmacol.Ther. *21*, 469-477.

Livesey,G., Johnson,I.T., Gee,J.M., Smith,T., Lee,W.E., Hillan,K.A., Meyer,J., and Turner,S.C. (1993). 'Determination' of sugar alcohol and Polydextrose absorption in humans by the breath hydrogen (H2) technique: the stoichiometry of hydrogen production and the interaction between carbohydrates assessed in vivo and in vitro. Eur.J.Clin.Nutr. *47*, 419-430.

Lomax,A.R. and Calder,P.C. (2008). Prebiotics, immune function, infection and inflammation: a review of the evidence. Br.J.Nutr. 1-26.

Louis,P., Scott,K.P., Duncan,S.H., and Flint,H.J. (2007). Understanding the effects of diet on bacterial metabolism in the large intestine. Journal of Applied Microbiology *102*, 1197-1208.

Macfarlane,G.T., Steed,H., and Macfarlane,S. (2008). Bacterial metabolism and health-related effects of galacto-oligosaccharides and other prebiotics. Journal of Applied Microbiology *104*, 305-344.

Mandalari,G., Nueno,P.C., Tuohy,K., Gibson,G.R., Bennett,R.N., Waldron,K.W., Bisignano,G., Narbad,A., and Faulds,C.B. (2007). In vitro evaluation of the prebiotic activity of a pectic oligosaccharide-rich extract enzymatically derived from bergamot peel. Appl.Microbiol.Biotechnol. *73*, 1173-1179.

Manhart,N., Spittler,A., Bergmeister,H., Mittlbock,M., and Roth,E. (2003). Influence of fructooligosaccharides on Peyer's patch lymphocyte numbers in healthy and endotoxemic mice. Nutrition *19*, 657-660.

Manichanh,C. *et al.* (2006). Reduced diversity of faecal microbiota in Crohn's disease revealed by a metagenomic approach. Gut *55*, 205-211.

Manzanares,W. and Hardy,G. (2008). The role of prebiotics and synbiotics in critically ill patients. Current Opinion in Clinical Nutrition and Metabolic Care *11*, 782-789.

Martin-Pelaez,S., Gibson,G.R., Martin-Orue,S.M., Klinder,A., Rastall,R.A., La Ragione,R.M., Woodward,M.J., and Costabile,A. (2008). In vitro fermentation of carbohydrates by porcine faecal inocula and their influence on Salmonella Typhimurium growth in batch culture systems. Fems Microbiology Ecology *66*, 608-619.

May,C.D. (1990). Industrial Pectins - Sources, Production and Applications. Carbohydrate Polymers *12*, 79-99.

McNeil,N.I. (1984). The contribution of the large intestine to energy supplies in man. Am.J.Clin.Nutr. *39*, 338-342.

Mead,P.S., Slutsker,L., Dietz,V., McCaig,L.F., Bresee,J.S., Shapiro,C., Griffin,P.M., and Tauxe,R.V. (1999). Food-related illness and death in the United States. Emerg.Infect.Dis. *5*, 607-625.

Mengaud,J., Ohayon,H., Gounon,P., Mege,R.-M., and Cossart,P. (1996). E-cadherin is the receptor for internalin, a surface protein required for entry of L. monocytogenes into epithelial cells. Cell *84*, 923-932.

Milenbachs,A.A., Brown,D.P., Moors,M., and Youngman,P. (1997). Carbon-source regulation of virulence gene expression in Listeria monocytogenes. Mol.Microbiol. *23*, 1075-1085.

Milohanic,E., Jonquieres,R., Cossart,P., Berche,P., and Gaillard,J.L. (2001). The autolysin Ami contributes to the adhesion of Listeria monocytogenes to eukaryotic cells via its cell wall anchor. Mol.Microbiol. *39*, 1212-1224.

Milohanic,E., Pron,B., Berche,P., and Gaillard,J.L. (2000). Identification of new loci involved in adhesion of Listeria monocytogenes to eukaryotic cells. European Listeria Genome Consortium. Microbiology *146 (Pt 3)*, 731-739.

Minami,Y., Yazawa,K., Tamura,Z., Tanaka,T., and Yamamoto,T. (1983). Selectivity of Utilization of Galactosyl-Oligosaccharides by Bifidobacteria. Chemical & Pharmaceutical Bulletin *31*, 1688-1691.

Mitsuoka,T., Hayakawa,K., and Kimura,N. (1974). [The faecal flora of man. II. The composition of bifidobacterium flora of different age groups (author's transl)]. Zentralbl.Bakteriol.Orig.A *226*, 469-478.

Moro,G., Minoli,I., Mosca,M., Fanaro,S., Jelinek,J., Stahl,B., and Boehm,G. (2002). Dosage-related bifidogenic effects of galacto- and fructooligosaccharides in formula-fed term infants. J.Pediatr.Gastroenterol.Nutr. *34*, 291-295.

Mountzouris,K.C., McCartney,A.L., and Gibson,G.R. (2002). Intestinal microflora of human infants and current trends for its nutritional modulation. British Journal of Nutrition *87*, 405-420.

O'Driscoll,B., Gahan,C.G., and Hill,C. (1996). Adaptive acid tolerance response in Listeria monocytogenes: isolation of an acid-tolerant mutant which demonstrates increased virulence. Appl.Environ.Microbiol. *62*, 1693-1698.

Olano-Martin,E., Gibson,G.R., and Rastall,R.A. (2002). Comparison of the in vitro bifidogenic properties of pectins and pectic-oligosaccharides. Journal of Applied Microbiology *93*, 505-511.

Ooi,S.T. and Lorber,B. (2005). Gastroenteritis due to Listeria monocytogenes. Clin.Infect.Dis. *40*, 1327-1332.

Pandiripally,V.K., Westbrook,D.G., Sunki,G.R., and Bhunia,A.K. (1999). Surface protein p104 is involved in adhesion of Listeria monocytogenes to human intestinal cell line, Caco-2. J.Med.Microbiol. *48*, 117-124.

Park,J.H., Lee,Y.S., Lim,Y.K., Kwon,S.H., Lee,C.U., and Yoon,B.S. (2000). Specific binding of recombinant Listeria monocytogenes p60 protein to Caco-2 cells. FEMS Microbiol.Lett. *186*, 35-40.

Park,S.F. (1994). The repression of listeriolysin O expression in Listeria monocytogenes by the phenolic beta-D-glucoside, arbutin. Lett.Appl.Microbiol. *19*, 258-260.

Park,S.F. and Kroll,R.G. (1993). Expression of listeriolysin and phosphatidylinositol-specific phospholipase C is repressed by the plant-derived molecule cellobiose in Listeria monocytogenes. Mol.Microbiol. *8*, 653-661.

Pentecost,M., Otto,G., Theriot,J.A., and Amieva,M.R. (2006). Listeria monocytogenes invades the epithelial junctions at sites of cell extrusion. PLoS.Pathog. *2*, e3.

Pentecost,M., Kumaran,J., Ghosh,P. and Amieva, M.R. (2010). Listeria monocytogenes Internalin B activates junctional endocytosis to accelerate intestinal invasion. PloS Pathogens. *6*. 1-15.

Petersen,A., Heegaard,P.M., Pedersen,A.L., Andersen,J.B., Sorensen,R.B., Frokiaer,H., Lahtinen,S.J., Ouwehand,A.C., Poulsen,M., and Licht,T.R. (2009). Some putative prebiotics increase the severity of Salmonella enterica serovar Typhimurium infection in mice. BMC Microbiol. *9*, 245.

Petkevicius,S., Thomsen,L.E., Knudsen,K.E.B., Murrell,K.D., Roepstorff,A., and Boes,J. (2007). The effect of inulin on new and on patent infections of *Trichuris suis* in growing pigs. Parasitology *134*, 121-127.

Pilgrim,S., Kolb-Maurer,A., Gentschev,I., Goebel,W., and Kuhn,M. (2003). Deletion of the gene encoding p60 in Listeria monocytogenes leads to abnormal cell division and loss of actin-based motility. Infect.Immun. *71*, 3473-3484.

Pirie,J.H.H. A new disease of veld rodents. "Tiger river disease". Publ.S.Afr.Inst.Med.Res. 3, 163-186. 1927.

Popowska,M. (2004). Analysis of the peptidoglycan hydrolases of Listeria monocytogenes: multiple enzymes with multiple functions. Pol.J.Microbiol. *53 Suppl*, 29-34.

Premaratne,R.J., Lin,W.J., and Johnson,E.A. (1991). Development of an improved chemically defined minimal medium for Listeria monocytogenes. Appl.Environ.Microbiol. *57*, 3046-3048.

Probert,H.M., Apajalahti,J.H.A., Rautonen,N., Stowell,J., and Gibson,G.R. (2004). Polydextrose, lactitol, and fructo-oligosaccharide fermentation by colonic bacteria in a three-stage continuous culture system. Applied and Environmental Microbiology *70*, 4505-4511.

Pron,B., Boumaila,C., Jaubert,F., Sarnacki,S., Monnet,J.P., Berche,P., and Gaillard,J.L. (1998). Comprehensive study of the intestinal stage of listeriosis in a rat ligated ileal loop system. Infect.Immun. *66*, 747-755.

Qin,J. *et al.* (2010). A human gut microbial gene catalogue established by metagenomic sequencing. Nature *464*, 59-65.

Rajilic-Stojanovic,M., Smidt,H., and de Vos,W.M. (2007). Diversity of the human gastrointestinal tract microbiota revisited. Environ.Microbiol. *9*, 2125-2136.

Rhoades,J., Gibson,G., Formentin,K., Beer,M., and Rastall,R. (2006). Inhibition of the adhesion of enteropathogenic Escherichia coli strains to HT-29 cells in culture by chito-oligosaccharides. Carbohydrate Polymers *64*, 57-59.

Rhoades,J., Manderson,K., Wells,A., Hotchkiss,A.T., Jr., Gibson,G.R., Formentin,K., Beer,M., and Rastall,R.A. (2008). Oligosaccharide-mediated inhibition of the adhesion of pathogenic Escherichia coli strains to human gut epithelial cells in vitro. J.Food Prot. *71*, 2272-2277.

Rijkers,G.T. *et al.* (2010). Guidance for substantiating the evidence for beneficial effects of probiotics: current status and recommendations for future research. J.Nutr. *140*, 671S-676S.

Roberfroid,M. (2005a). Inulin-Type Fructans and the Defence Functions of the Body. In Ira Wolinsky (Ed.). Inulin-Type Fructans.

Roberfroid,M. (2007). Prebiotics: the concept revisited. J.Nutr. *137*, 830S-837S.

Roberfroid,M. (2005b). The Digestive Functions: Inulin-Type Fructans As Fermentable Carbohydrates: In Ira Wolinsky (Ed.). Inulin-Type Fructans. CRC Press.

Roberfroid,M. (2005c). The Gastrointestinal System: In Ira Wolinsky (Ed.). Inulin-Type Fructans. CRC Press LLC.

Roberfroid,M. (1993). Dietary fiber, inulin, and oligofructose: a review comparing their physiological effects. Crit Rev.Food Sci.Nutr. *33*, 103-148.

Roberfroid,M.B. (2008a). General Introduction: Prebiotics in Nutrition. In: Handbook of Prebiotics, ed. G.R.Gibson and M.Roberfroid CRC Press, 1-11.

Roberfroid,M.B. (2005d). Introducing inulin-type fructans. Br.J.Nutr. *93 Suppl 1*, S13-S25.

Roberfroid,M.B. (2008b). Prebiotics: Concept, Definition, Criteria, Methologies, and Products. In: Handbook of Prebiotics, ed. G.R.Gibson and M.Roberfroid CRC Press, 39-68.

Rossi,M., Corradini,C., Amaretti,A., Nicolini,M., Pompei,A., Zanoni,S., and Matteuzzi,D. (2005). Fermentation of fructooligosaccharides and inulin by bifidobacteria: a comparative study of pure and fecal cultures. Appl.Environ.Microbiol. *71*, 6150-6158.

Rowland,I.R. and Tanaka,R. (1993). The effects of transgalactosylated oligosaccharides on gut flora metabolism in rats associated with a human faecal microflora. J.Appl.Bacteriol. *74*, 667-674.

Ruas-Madiedo,P., Gueimonde,M., de los Reyes-Gavilan CG, and Salminen,S. (2006). Short communication: effect of exopolysaccharide isolated from "viili" on the adhesion of probiotics and pathogens to intestinal mucus. J.Dairy Sci. *89*, 2355-2358.

Ruhland,G.J., Hellwig,M., Wanner,G., and Fiedler,F. (1993). Cell-surface location of Listeria-specific protein p60--detection of Listeria cells by indirect immunofluorescence. J.Gen.Microbiol. *139*, 609-616.

Saavedra,J., Tschernia,A., and Moore,N. (1999). Gastrointestinal function in infants consuming a weaing food supplemented with oligofructose, a prebiotic. Journal of Pediatric and Gastroenterology Nutrition *29*, 95.

Santiago,N.I., Zipf,A., and Bhunia,A.K. (1999). Influence of temperature and growth phase on expression of a 104-kilodalton Listeria adhesion protein in Listeria monocytogenes. Appl.Environ.Microbiol. *65*, 2765-2769.

Searle,L.E. *et al.* (2009). A mixture containing galactooligosaccharide, produced by the enzymic activity of Bifidobacterium bifidum, reduces Salmonella enterica serovar Typhimurium infection in mice. J.Med.Microbiol. *58*, 37-48.

Servin,A.L. (2004). Antagonistic activities of lactobacilli and bifidobacteria against microbial pathogens. FEMS Microbiol.Rev. *28*, 405-440.

Shen,Y., Naujokas,M., Park,M., and Ireton,K. (2000). InIB-dependent internalization of Listeria is mediated by the Met receptor tyrosine kinase. Cell *103*, 501-510.

Shoaf,K., Mulvey,G.L., Armstrong,G.D., and Hutkins,R.W. (2006). Prebiotic galactooligosaccharides reduce adherence of enteropathogenic Escherichia coli to tissue culture cells. Infect.Immun. *74*, 6920-6928.

Slavica,V.M., Obradovic,D., Velebit,B., Branka,B., Marija,S., and Turubatovic,L. (2010). Antimicrobial Properties of Indigenous Lactobacillus Sakei Strain. Acta Veterinaria-Beograd *60*, 59-66.

Sleator,R.D., Watson,D., Hill,C., and Gahan,C.G. (2009). The interaction between Listeria monocytogenes and the host gastrointestinal tract. Microbiology *155*, 2463-2475.

Smith,G.A., Portnoy,D.A., and Theriot,J.A. (1995). Asymmetric distribution of the Listeria monocytogenes ActA protein is required and sufficient to direct actin-based motility. Mol.Microbiol. *17*, 945-951.

Sokol,H. *et al.* (2008). Faecalibacterium prausnitzii is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients. Proc.Natl.Acad.Sci.U.S.A *105*, 16731-16736.

Soltys,B.J. and Gupta,R.S. (1997). Cell surface localization of the 60 kDa heat shock chaperonin protein (hsp60) in mammalian cells. Cell Biol.Int. *21*, 315-320.

Stephen,A.M. and Cummings,J.H. (1980). The microbial contribution to human faecal mass. J.Med.Microbiol. *13*, 45-56.

Suarez,M., Gonzalez-Zorn,B., Vega,Y., Chico-Calero,I., and Vazquez-Boland,J.A. (2001). A role for ActA in epithelial cell invasion by Listeria monocytogenes. Cell Microbiol. *3*, 853-864.

Swanson,J.A. and Baer,S.C. (1995). Phagocytosis by zippers and triggers. Trends Cell Biol. *5*, 89-93.

Takahashi,T., Karita,S., Yahaya,M.S., and Goto,M. (2005). Radial and axial variations of bacteria within the cecum and proximal colon of guinea pigs revealed by PCR-DGGE. Biosci.Biotechnol.Biochem. *69*, 1790-1792.

Ten Bruggencate,S.J., Bovee-Oudenhoven,I.M., Lettink-Wissink,M.L., Katan,M.B., and Van der,M.R. (2004). Dietary fructo-oligosaccharides and inulin decrease resistance of rats to salmonella: protective role of calcium. Gut *53*, 530-535.

Ten Bruggencate,S.J., Bovee-Oudenhoven,I.M., Lettink-Wissink,M.L., and Van der,M.R. (2003). Dietary fructo-oligosaccharides dose-dependently increase translocation of salmonella in rats. J.Nutr. *133*, 2313-2318.

Ten Bruggencate,S.J., Bovee-Oudenhoven,I.M., Lettink-Wissink,M.L., and Van der,M.R. (2005). Dietary fructooligosaccharides increase intestinal permeability in rats. J.Nutr. *135*, 837-842.

Tschernia,A., Moore,N., and Abi-hanna,A. (1999). Effects of long-term supplementation of a weaning food supplememted with oligofructose a prebiotic, on general infant health status. Journal of Pediatric and Gastroenterology Nutrition *29*, 58.

Tuohy,K.M., Kolida,S., Lustenberger,A.M., and Gibson,G.R. (2001). The prebiotic effects of biscuits containing partially hydrolysed guar gum and fructo-oligosaccharides--a human volunteer study. Br.J.Nutr. *86*, 341-348.

Tuomola,E.M., Ouwehand,A.C., and Salminen,S.J. (1999). The effect of probiotic bacteria on the adhesion of pathogens to human intestinal mucus. FEMS Immunol.Med.Microbiol. *26*, 137-142.

Tzortzis,G., Goulas,A.K., Gee,J.M., and Gibson,G.R. (2005). A novel galactooligosaccharide mixture increases the bifidobacterial population numbers in a continuous in vitro fermentation system and in the proximal colonic contents of pigs in vivo. J.Nutr. *135*, 1726-1731.

Vael,C. and Desager,K. (2009). The importance of the development of the intestinal microbiota in infancy. Curr.Opin.Pediatr. *21*, 794-800.

VanGijsegem,F. (1997). In planta regulation of phytopathogenic bacteria virulence genes: Relevance of plant-derived signals. European Journal of Plant Pathology *103*, 291-301.

Vanmaele,R.P., Heerze,L.D., and Armstrong,G.D. (1999). Role of lactosyl glycan sequences in inhibiting enteropathogenic Escherichia coli attachment. Infection and Immunity *67*, 3302-3307.

varez-Dominguez,C., Vazquez-Boland,J.A., Carrasco-Marin,E., Lopez-Mato,P., and Leyva-Cobian,F. (1997). Host cell heparan sulfate proteoglycans mediate attachment and entry of Listeria monocytogenes, and the listerial surface protein ActA is involved in heparan sulfate receptor recognition. Infect.Immun. *65*, 78-88.

Vaughan,E.E., de Vries,M.C., Zoetendal,E.G., Ben-Amor,K., Akkermans,A.D., and de Vos,W.M. (2002). The intestinal LABs. Antonie Van Leeuwenhoek *82*, 341-352.

Vazquez,M.J., Alonso,J.L., Dominguez,H., and Parajo,J.C. (2000). Xylooligosaccharides: manufacture and applications. Trends in Food Science & Technology *11*, 387-393.

Vazquez-Boland,J.A., Kuhn,M., Berche,P., Chakraborty,T., Dominguez-Bernal,G., Goebel,W., Gonzalez-Zorn,B., Wehland,J., and Kreft,J. (2001). Listeria pathogenesis and molecular virulence determinants. Clin.Microbiol.Rev. *14*, 584-640.

Vieira,L.Q., dos Santos,L.M., Neumann,E., da Silva,A.P., Moura,L.N., and Nicoli,J.R. (2008). Probiotics protect mice against experimental infections. J.Clin.Gastroenterol. *42 Suppl 3 Pt 2*, S168-S169.

Voragen,A.G.J. (1998). Technological aspects of functional food-related carbohydrates. Trends in Food Science & Technology *9*, 328-335.

Waligora-Dupriet,A.J., Campeotto,F., Nicolis,I., Bonet,A., Soulaines,P., Dupont,C., and Butel,M.J. (2007). Effect of oligofructose supplementation on gut microflora and well-being in young children attending a day care centre. Int.J.Food Microbiol. *113*, 108-113.

Wampler,J.L., Kim,K.P., Jaradat,Z., and Bhunia,A.K. (2004). Heat shock protein 60 acts as a receptor for the Listeria adhesion protein in Caco-2 cells. Infect.Immun. *72*, 931-936.

Wang,X. and Gibson,G.R. (1993). Effects of the in vitro fermentation of oligofructose and inulin by bacteria growing in the human large intestine. J.Appl.Bacteriol. *75*, 373-380.

Watzl,B., Girrbach,S., and Roller,M. (2005). Inulin, oligofructose and immunomodulation. British Journal of Nutrition *93*, S49-S55.

Wells,A.L., Saulnier,D.M.A., and Gibson,G.R. (2008). Gastrointestinal Microflora and Interactions With Gut Mucosa. In: Handbook of Prebiotics, ed. Glenn R.Gibson and Marcel B.Roberfroid CRC Press, 13-38.

Werbrouck,H., Grijspeerdt,K., Botteldoorn,N., Van,P.E., Rijpens,N., Van,D.J., Uyttendaele,M., Herman,L., and Van,C.E. (2006). Differential inlA and inlB expression and interaction with human intestinal and liver cells by Listeria monocytogenes strains of different origins. Appl.Environ.Microbiol. *72*, 3862-3871.

Wolf,B., Meulbroek,J., Jarvis,K., Wheeler,K., and Garleb,K. (1997). Dietary supplementation with fructooligosaccharides increase survival time in an hamster model of Clostridium difficile. Bioscience Microflora *16*, 59-64.

Woodmansey,E.J., McMurdo,M.E., Macfarlane,G.T., and Macfarlane,S. (2004). Comparison of compositions and metabolic activities of fecal microbiotas in young adults and in antibiotic-treated and non-antibiotic-treated elderly subjects. Appl.Environ.Microbiol. *70*, 6113-6122.

Yanabe,M., Shibuya,M., Gonda,T., Asai,H., Tanaka,T., Sudou,K., Narita,T., Matsui,T., and Itoh,K. (2001). Establishment of specific pathogen-free guinea-pig colonies using limited-flora guinea-pigs associated with conventional guinea-pig flora, and monitoring of their cecal flora. Exp.Anim *50*, 105-113.

Zamfir,M., Callewaert,R., Cornea,P.C., Savu,L., Vatafu,I., and De Vuyst,L. (1999). Purification and characterization of a bacteriocin produced by Lactobacillus acidolphilus IBB 801. Journal of Applied Microbiology *87*, 923-931.

Zhong,J., Luo,B.Y., Xiang,M.J., Liu,H.W., Zhai,Z.K., Wang,T.S., and Craig,S.A.S. (2000). Studies on the effects of polydextrose intake on physiologic functions in Chinese people. American Journal of Clinical Nutrition *72*, 1503-1509.

Manuscript I

Certain dietary carbohydrates promote *Listeria* infection in a guinea pig model, while others prevent it

Published in International Journal of Food Microbiology

International Journal of Food Microbiology 140 (2010) 218–224

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Certain dietary carbohydrates promote *Listeria* infection in a guinea pig model, while others prevent it

Tine Ebersbach [a,*], Julie Boeck Jørgensen [a], Peter Michael Heegaard [b], Sampo J. Lahtinen [c], Arthur C. Ouwehand [c], Morten Poulsen [a], Hanne Frøkiær [d], Tine Rask Licht [a]

[a] National Food Institute, Technical University of Denmark, Mørkhøj Bygade 19, 2860 Soeborg, Denmark
[b] National Veterinary Institute, Technical University of Denmark, Bülowsvej 27, 1790 Copenhagen V, Denmark
[c] Danisco Finland, Health & Nutrition, Sokeritehtaantie 20, 02460 Kantvik, Finland
[d] Department of Basic Sciences and Environment Faculty of Life Sciences, Copenhagen University Thorvaldsensvej 40, 1871 Frederiksberg C, Denmark

ARTICLE INFO

Article history:
Received 9 November 2009
Received in revised form 14 January 2010
Accepted 16 March 2010

Keywords:
Listeria monocytogenes
Prebiotic
Guinea pig
Prevention of infection

ABSTRACT

It has been proposed that dietary non-digestible carbohydrates can improve host resistance to intestinal infections by stimulating health-promoting bacteria in the gut. However, evidence from *in vivo* infection studies is scarce, particularly for gram-positive infections. We studied the effect of five non-digestible carbohydrates on the resistance of guinea pigs to *Listeria monocytogenes* infections. Animals were fed a diet supplemented with 10% xylooligosaccharides (XOS), galactooligosaccharides (GOS), inulin, apple pectin or polydextrose for three weeks before oral infection with a mixture of three different fluorescently labeled *L. monocytogenes* strains. Colonisation of *L. monocytogenes* in the intestine was determined by quantification of *L. monocytogenes* in faecal, ileal and caecal samples while translocation was determined by quantification of *L. monocytogenes* in mesenteric lymph nodes, spleen and liver. XOS and GOS significantly ($P<0.05$) improved the resistance of guinea pigs to *L. monocytogenes*, while inulin and apple pectin decreased the resistance ($P<0.05$). No significant effect on resistance to *L. monocytogenes* was seen after feeding with polydextrose. No difference in caecal weight or pH between the dietary groups was measured, except for a higher caecal weight and a lower caecal pH of animals fed with XOS, and a lower caecal pH for animals fed with polydextrose. In conclusion, this study shows for the first time that different non-digestible carbohydrates can have entirely different effects on the intestinal colonisation and translocation of a pathogenic bacterium.

1. Introduction

Gastrointestinal infections induced by food borne pathogens are a major clinical problem worldwide. Furthermore, the growing resistance of pathogenic bacteria to antibiotics provides additional rationale for developing therapies aiming at prevention and treatment of diseases caused by food borne pathogens. Certain dietary non-digestible carbohydrates (prebiotics) selectively stimulate the growth of the health-promoting bacteria in the gut and thereby potentially prevent or moderate intestinal infections (Gibson et al., 1995;Kaplan and Hutkins, 2000). Prebiotics are defined as selectively fermented food ingredients that allow specific changes in the composition and/or activity of the gastrointestinal microbiota, which confers benefits upon host well-being and health (Roberfroid, 2007).

Indigenous bifidobacteria and lactobacilli are believed to play an important role for human health and immune function, and an increase in the number of these bacteria is assumed to repress pathogen colonisation in the intestine (Orrhage and Nord, 2000). Several studies both *in vitro* and *in vivo* have shown that dietary prebiotics including inulin and oligofructose selectively stimulate growth of intestinal bifidobacteria as well as certain *Lactobacillus* strains (Wang and Gibson, 1993; Gibson et al., 1995; Tuohy et al., 2001). There are a number of potential mechanisms through which bifidobacteria and lactobacilli may moderate intestinal infection. Firstly, both bifidobacteria and lactobacilli produce antimicrobial compounds such as bacteriocins that are toxic for some pathogens including *Listeria* (Gibson and Wang, 1994; Drider et al., 2006). Additionally, lactic acid bacteria (LAB) produce lactic acid as the major metabolic end-product of fermentation and thereby lower the pH in the intestine to a level that is unfavourable for most pathogenic bacteria. Other mechanisms include increased competition for nutrients and blocking of mucosal adhesion sites in the intestine (Shoaf et al., 2006; Rhoades et al., 2008). In withstanding the first encounter with a pathogen, these initial non-immunological host defences are especially important. Furthermore, evidence is accumulating that some prebiotics execute a positive stimulation of the immune system mainly on immunological processes taking place at

* Corresponding author. Tel.: +45 35 88 7091.
E-mail address: tiebe@food.dtu.dk (T. Ebersbach).

0168-1605/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijfoodmicro.2010.03.030

the level of the gut-associated lymphoid tissue (for a review see (Seifert and Watzl, 2007)). Immuno-modulatory effects of prebiotics may be modulated by prebiotic-induced changes in the composition of intestinal microbes, which in turn interact with gut-associated lymphoid tissue.

Still, compelling scientific evidence from *in vivo* infection studies proving that dietary non-digestible carbohydrates increase host resistance to intestinal bacterial infections is lacking. Only few *in vivo* studies have so far directly investigated the effect of prebiotic carbohydrates against pathogenic bacterial infections and most of them have used gram-negative bacteria as the infective agent (Lomax and Calder, 2008).

The aim of the present study was to determine the effect of dietary non-digestible carbohydrates on the resistance of guinea pigs to intestinal colonisation and translocation of the gram-positive *Listeria monocytogenes*. *L. monocytogenes* causes the disease listeriosis, which is acquired by ingesting contaminated food products. Severe listeriosis mainly affects pregnant women, newborn and immuno-compromised patients. *L. monocytogenes* infections are not frequent, but with a mean mortality rate of 20–30%, they are among the most deadly food borne infections in industrialised countries (Vazquez-Boland et al., 2001). *L. monocytogenes* does not interact optimally with the specific intestinal epithelial receptor of mice or rats, and these otherwise frequently used rodents are therefore not suitable as models for human listerial infection. In contrast, guinea pigs possess an intestinal receptor analogous to that of humans, and a guinea pig model (Roldgaard et al., 2009) was used in this study. The model was based on fluorescent labeled *Listeria*, and allowed infection with a mixture of three different *L. monocytogenes* strains, including a food isolate, a clinical isolate, and the reference strain ScottA. This was considered a feasible approach since several reports indicate that interactions between given carbohydrates and given bacteria can be very strain dependent (Palframan et al., 2003; Vernazza et al., 2006), and the applied model has previously proved useful for comparison of *L. monocytogenes* strains (Andersen et al., 2006; Roldgaard et al., 2009).

The non-digestible carbohydrates tested in this study were inulin with a degree of polymerisation (DP) of 2–60, apple pectin with a DP greater than 100 (Vos et al., 2007; Mandalari et al., 2007), xylooligosaccharides (XOS) with a DP of 2–10, galactoligosaccharides (GOS) with a DP of 2–6 and polydextrose with an average DP of 12.

2. Materials and methods

2.1. Bacterial strains, plasmids and media

Three *L. monocytogenes* isolates were used: Strain 7291, serogroup 4, isolated from a chicken pasta salad, the clinical isolate 4446, serogroup 4, isolated from a 63-year-old female with septicaemia (Larsen et al., 2002), and the clinical reference isolate ScottA. All three strains carried nalidixic acid resistance. All *L. monocytogenes* strains were labeled with either cyan fluorescent protein (CFP), yellow fluorescent protein (YFP) or DsRedExpress (DsR) as previously described (Andersen et al., 2006). The plasmids carried erythromycin resistance. All of the nine (3×3) possible strain/label combinations were used. Bacteria were cultivated on brain heart infusion (BHI) agar (Oxoid) or in liquid BHI (Oxoid) and when appropriate nalidixic acid (Sigma) or erythromycin (Sigma) was used at a final concentration of 100 µg/ml or 10 µg/ml, respectively. To verify the absence of *Listeria* before dosage, faecal samples from the guinea pigs were tested by plating on Rapid *L. mono* plates (BioRad).

2.2. Preparation of inocula for oral dosage

Inocula for oral dosage were prepared as described earlier (Roldgaard et al., 2009) with the following modifications: Overnight cultures of the three *L. monocytogenes* strains were diluted to an $OD_{600} = 0.01$ in BHI supplemented with nalidixic acid and erythromycin. To obtain oxygen-restricted cultures which are more infectious (Andersen et al., 2007), atmospheric air above the diluted cultures was exchanged with sterile nitrogen and the lids of the bottles were tightened and sealed. The cultures were incubated at 37 °C in a rotary shaker at 200 rpm for approximately 20 h (approximately $OD_{600} = 1.1$). Of each of the three cultures, 500 ml was harvested by centrifugation (1800×*g*, 25 min, 22 °C) and the three pellets were pooled and resuspended in double cream (38% fat, pH 7) to a final volume of 12 ml. The cream suspension of the three *Listeria* strains was immediately used for dosage. Samples of the inocula were diluted and spread onto BHI-agar supplemented with nalidixic acid and erythromycin to estimate the number of the respective *L. monocytogenes* strains present in the double cream suspension. Each animal was dosed with 0.75 ml of a cream/*Listeria* suspension containing approximately a total of 10^{11} *L. monocytogenes* represented by approximately 3×10^{10} bacteria of each of the three *Listeria* strains.

2.3. Animals and housing

Equal numbers of male and female Hartley guinea pigs (Charles River Laboratories, Germany) weighing 260 g (± 10 g), aged 3 weeks, were used. The animals were randomised according to weight and gender and housed individually in polycarbonate cages, Eurostandard Type IV (1820 cm^2) with raised lid (total height 27 cm) and tapvei bedding (peeled Aspen hardwood, Tapvei Kaavi, Finland). During the study the temperature was maintained at 22 ± 5%, air was changed 8–10 times per hour, and the light was on from 9.00 a.m. to 9.00 p.m.

2.4. Diets and experimental design

Three animal experiments were carried out. In Experiment A, inulin (eight animals) and apple pectin (eight animals) were tested, in Experiment B XOS (six animals) and GOS (seven animals) were tested and in Experiment C polydextrose (eight animals) was tested. In each of the three experiments, a control group of seven or eight animals was included. During a period of two weeks the animals were habituated into eating a diet (guinea pig diet C3000, Altromin, Lage, Germany) containing 18.5% of undefined polysaccharides as the main carbohydrate source, or a diet in which 10% of the polysaccharides were constituted by one of the indigestible carbohydrates, while the remaining 8.5% were similar to the carbohydrate source present in the control diet (Table 1).

Diets contained the same amounts of macro- and micronutrients. The animals were subsequently fed their respective diets for three and a half weeks before they were transferred to polycarbonate cages in negatively pressurised isolators due to safety regulations. All animals, both in the control groups and the groups given prebiotics were dosed with the cream/*Listeria* suspension on Day 0 and again on Day 1. The animals were starved for 24 h before the first dosage. Dosage was carried out directly in the oral cavity, between the incisors and the molars which prevents spillage. Following dosage, the animals had

Table 1
Composition of the diets. Only modifications from the original C3000 diet are listed. All numbers are in g/kg. [a]Fed with unmodified C3000.

Group	Control[a]	Inulin	Apple pectin	XOS	GOS	Polydextrose
Inulin	0	100	0	0	0	0
Apple pectin	0	0	100	0	0	0
XOS	0	0	0	100	0	0
GOS	0	0	0	0	100	0
Polydextrose	0	0	0	0	0	100
Undefined polysaccharide	185	85	85	85	85	85
Vitamin C	2	2	2	2	2	2

access to food and water *ad libitium* throughout the duration of the study. Animal weight was measured each week throughout the experiment. Fresh faecal samples were collected every day. Half of the animals were euthanised on Day 4, and the other half on Day 7, except in a few cases (described in the Result section) where euthanisation prior to Day 4 was necessary due to ethical considerations. Ileal and caecal content and samples from the liver, spleen and mesenteric lymph nodes were collected and caecal weights and pH were recorded for each animal. Blood samples were collected a couple of days before the first dosage with *Listeria* and just before the animals were euthanised.

Inulin (BENEO ST-gel, purity >90%) was purchased from Alsiano (Birkerød, Denmark), apple pectin (Brown Ribbon Pure, food grade purity) and XOS (purity >90%) were kindly provided by Obipektin AG (Bischofszell, Switzerland) and Lenzing AG, Lenzing, Austria respectively, while GOS (food grade purity) and polydextrose (Litesse Ultra®, food grade purity) were kindly provided by Danisco Limited, Redhill, United Kingdom.

2.5. Ethical aspects

All animal experiments were carried out under the supervision of the Danish National Agency of Experimental Animals. The applied guinea pig model (Roldgaard et al., 2009) is less stressful to the guinea pigs than previously published models (Lecuit et al., 2001). Additionally, the number of animals needed is reduced because each animal is treated with a mixture of different strains. The animals were observed twice a day and if an animal displayed severe signs of infection (ruffled fur, appetite loss, and reduced activity), it was euthanised.

2.6. Selective cultivation

L. monocytogenes from faecal, ileal and caecal samples were enumerated on BHI agar supplemented with erythromycin and nalidixic acid. Liver, spleen and mesenteric lymph nodes were homogenised using mortar and pestle, and numbers of *L. monocytogenes* were determined by plating of homogenised, diluted tissue on BHI agar supplemented with erythromycin and nalidixic acid. After 48–72 h of incubation at 37 °C, the plates were placed on a UV table (excitation at 302 nm), and fluorescent colonies (either CFP, YFP or DsR) were enumerated.

2.7. In vitro fermentation

The ability of the three *L. monocytogenes* strains used in this study to grow anaerobically on the five prebiotics and glucose (positive control) was tested. Overnight cultures of *L. monocytogenes* 4446, *L monocytogenes* 7291 and *L. monocytogenes* ScottA grown in BHI were diluted to an $OD_{600} = 0.01$ in basal medium (g/l): Peptone water (Oxoid) 2; NaCl 0.1; K_2HPO_4 0.04; KH_2PO 0.04; $MgSO_47H_2O$ 0.01; $CaCl_26H_2O$ 0.01; $NaHCO_3$ 2 were autoclaved before sterile filtrations of L-CYSTEINE HCl (Sigma) 0.5; yeast extract (Oxoid) 2; haemin (Sigma) 0.005; tween 80 (Merck) 2; vitamin K_1 (Sigma) 10; bile salts (Oxiod) 0.5; resazurin (Sigma) 0.001; and 1% (w/v) of one of the five test prebiotics or glucose was added. The initial pH in the sample was measured. Atmospheric air above the diluted cultures was exchanged with sterile nitrogen and the lids of the bottles were tightened and sealed. After 24 h of anaerobic growth, OD_{600} and the pH in the sample was measured. Each species was grown on each carbohydrate in triplicate. In order to determine the growth of the strains on the basal medium alone, medium with no added carbohydrate was inoculated for each species in triplicate. Growth was defined as a significantly ($P>0.05$) larger optical density in the sample supplemented with the carbohydrate when compared to the blank sample.

2.8. Acute phase protein measurements

A commercially available sandwich ELISA assay (Phase SAA assay, Tridelta Development Ltd., Kildare, Ireland) was used for determination of Serum Amyloid A (SAA) in blood samples from the guinea pigs. This assay is based on anti-human monoclonal antibodies in a sandwich set-up as originally described by McDonald et al. (1991).

The assay proved to be cross-reactive with guinea pig SAA, however no guinea pig SAA standard was available for quantification and instead the porcine standard of the kit was used.

Samples were tested according to the manufacturer's instructions except that the lowest dilution was 1:10 to increase signal intensity. The detection limit of the assay was 1.25 mg/l expressed in porcine SAA equivalents.

To our knowledge this is the first demonstration of the acute phase response of a guinea pig homologue of SAA. Further characterisation of guinea pig SAA will be described in a separate paper (Heegaard et al. paper in preparation).

2.9. Specific antibodies against Listeria

Antibodies against *Listeria* in blood samples from the animals were measured by indirect ELISA (Fonnesbech et al., 1993). Briefly, microtiter plates (Maxisorp, NUNC, Roskilde, Denmark) were coated with a mixture of equal amounts of the three *Listeria* isolates taken from UV-killed overnight cultures and diluted 25 times to obtain optimal binding of antibodies. Blood samples taken before and 7 days after oral dosage were two-fold diluted from 27 to 2212 times in PBS-tween 20 buffer and the titer determined.

2.10. Statistics

All statistical tests were performed using the statistical software in Microsoft Office Excel XP (Microsoft, USA). A one-way analysis of variance was used on all measured parameters to determine the effect of diet. Tests were considered statistically significant if P values <0.05 were obtained.

Similarly, a one-way analysis of variance was used to determine if data from different experiments could be pooled. Data were pooled only if P values >0.05 were obtained.

3. Results

3.1. Presence of L. monocytogenes in faecal samples

Faecal contents of *L. monocytogenes* ranged between 10^5 and 10^8 CFU/g faeces on Day 1 after the first dosage and decreased to between 10^4 and 10^6 CFU/g feaces on Day 7 (data not shown). No significant difference in faecal *L. monocytogenes* counts was seen between the different groups. Feeding with non-digestible carbohydrates did not result in a different distribution between the three *L. monocytogenes* strains (7291, 4446 and ScottA) than was observed in the control groups. As previously reported (Roldgaard et al., 2009), strain 7291 was in all cases seen to be slightly more predominant in faecal samples than strain 4446 and ScottA. No difference between strain 4446 and ScottA was observed (data not shown).

No difference was observed between female and male animals. As previously reported (Andersen et al., 2007), labeling of *L. monocytogenes* cells with CFP, YFP or DsR had no influence on the infectivity of the cells (data not shown).

3.2. Effects on caecal weight and pH

Within each experiment, no significant difference in animal weight between each feeding group and the control group was recorded at the time of infection (data not shown). However, the

average caecum weight differed between control groups within each experiment (Table 2), probably due to differences between batches of animals.

No difference in caecal weight between dietary groups was seen except in Experiment B where animals fed with XOS had a significantly larger caecal weight ($P<0.05$) than recorded for the control group. Consistently, also the caecal pH was significantly lower ($P<0.005$) in animals fed with XOS. Animals fed with polydextrose had lower pH in caecum ($P<0.005$), but not a significantly lower caecal weight (Table 2). Even though these pH drops were significant, they were quite modest — animals fed with XOS and polydextrose both had a pH of approximately 6.8 and the control groups a pH of 7.19.

3.3. Presence of *L. monocytogenes* in organs

Statistical analysis revealed that within each of the three experiments A, B, and C, there were no differences between organ counts of *L. monocytogenes* obtained on Day 4 and Day 7 post infection, thus data from the different days were pooled. As in the faecal samples, strain 7291 was both in the intestine and in the organs slightly more predominant than strain 4446 and ScottA. No difference between strain 4446 and ScottA was observed (data not shown). Feeding with non-digestible carbohydrates did not result in a different distribution in the intestine or relative translocation to the organs between the three *L. monocytogenes* strains (7291, 4446 and ScottA) than was observed in the control groups. Therefore, data from the three strains were pooled.

Experiment A (23 animals): Numbers of *Listeria* in organs of animals fed a diet containing inulin were significantly higher ($P<0.05$) in caecum ($\log(CFU)=7.2$) and spleen ($\log(CFU)=3.9$) when compared to animals fed with the control diet (caecum log ($CFU)=5.1$; spleen $\log(CFU)=2.0$), while animals fed with apple pectin had significantly higher ($P<0.05$) amounts of *Listeria* in liver ($\log(CFU)=4.5$) and spleen ($\log(CFU)=3.6$) than the control group (liver $\log(CFU)=2.6$; spleen $\log(CFU)=2.0$) (Fig. 1). *Listeria* was recovered from the livers of all animals fed with inulin or apple pectin and only from three out of seven animals in the control group (Table 3). Consistently, *Listeria* was recovered from the spleen from all animals fed inulin and from seven out of eight animals fed with apple pectin. Only four out of seven animals in the control group had *Listeria* in the spleen. Some of the animals in experiment A had to be euthanised due to ethical considerations one or two days earlier than planned, because they developed symptoms of severe disease (ruffled fur and changed behaviour). This is true for two animals in the control group, four animals in the inulin-fed group and one animal in the pectin-fed group. Organ counts from these animals were pooled with the rest.

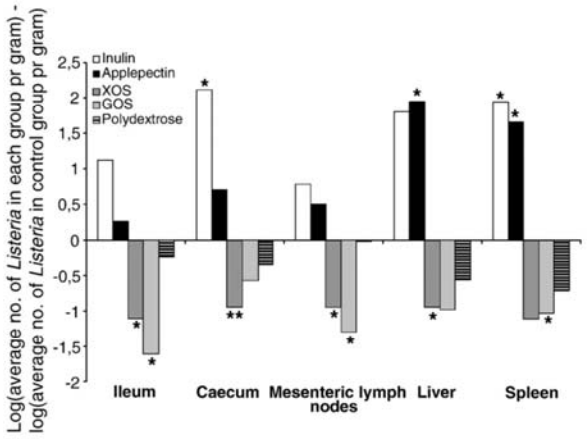


Fig. 1. Log(average number of *Listeria* in each group pr gram)−log(average number of *Listeria* in control group pr gram) from experiments A, B and C. Samples were taken either 4 or 7 days post first infection (data pooled). Values significantly different from the control group are marked by stars. *: $P<0.05$; **: $P<0.005$. When no *Listeria* was detected, a value of half of the detection limit was inserted.

In the following two experiments, no animals were euthanised pre-term, probably due to variations between individual batches of animals or to differences in the subjective evaluation of the symptoms leading to euthanisation.

Experiment B (21 animals): In animals fed a diet containing XOS, significantly fewer ($P<0.05$) *Listeria* were recovered from ileum (log ($CFU)=3.9$), caecum ($\log(CFU)=3.9$), mesenteric lymph nodes (log ($CFU)=3.4$) and liver samples ($\log(CFU)=1.7$) than the control group (ileum $\log(CFU)=5.1$; caecum $\log(CFU)=4.9$; lymph nodes $\log(CFU)=4.3$; liver $\log(CFU)=2.7$). Similarly, feeding with GOS resulted in significantly fewer ($P<0.05$) listerial cells in ileum (log ($CFU)=3.4$), mesenteric lymph nodes ($\log(CFU)=3.0$) and in the spleen ($\log(CFU)=1.9$; control $\log(CFU)=3.0$) (Fig. 1). Consistently, translocation of *Listeria* to liver and spleen was observed in fewer of these animals than in animal fed the control diet (Table 3).

Experiment C (16 animals): The occurrence and numbers of *Listeria* found in the intestine (ileum $\log(CFU)=3.8$; caecum $\log(CFU)=3.8$) and organs (lymph nodes $\log(CFU)=3.8$; liver $\log(CFU)=1.6$; spleen $\log(CFU)=1.5$) of animals fed a diet supplemented with polydextrose were not significantly different from the control (ileum $\log(CFU)=4.0$; caecum $\log(CFU)=4.1$; lymph nodes $\log(CFU)=3.8$; liver $\log(CFU)=2.1$; spleen $\log(CFU)=2.2$) (Table 3 and Fig. 1).

There was no statistically significant difference between the numbers of *Listeria* recovered from the organs of the guinea pigs in the three control groups from experiments A, B, and C. However, the number of animals in which this pathogen was detected in liver and spleen was lower in the control group of experiment A, than in the

Table 2
Weight of the caecum in proportion to animal weight and pH in the caecum measured at the time of euthanisation are followed by standard error of the mean of each group. Values significantly different from the control group are marked by stars. *: $P<0.05$; **: $P<0.005$.

Experiment	Group	Weight of the caecum in proportion to animal weight (%)	pH in caecum
A	Control	12.1 ± 2.5	7.28 ± 0.08
	Inulin	11.0 ± 1.9	7.19 ± 0.09
	Apple pectin	14.2 ± 3.4	7.25 ± 0.08
B	Control	5.7 ± 0.8	7.19 ± 0.07
	XOS	8.4 ± 0.5*	6.80 ± 0.07**
	GOS	8.1 ± 1.1	7.18 ± 0.18
C	Control	9.1 ± 1.0	7.19 ± 0.10
	Polydextrose	8.2 ± 0.8	6.83 ± 0.03**

Table 3
Number of guinea pigs from experiment A, B and C where *L. monocytogenes* was recovered from ileum, caecum, mesenteric lymph nodes, liver and spleen after oral dosage with *L. monocytogenes*.

Experiment		Ileum	Caecum	Mesentericl lymph nodes	Liver	Spleen
A	Control	7/7	7/7	7/7	3/7	4/7
	Inulin	8/8	8/8	8/8	8/8	8/8
	Apple pectin	7/8	7/8	8/8	8/8	7/8
B	Control	8/8	8/8	8/8	7/8	8/8
	XOS	6/6	6/6	6/6	2/6	4/6
	GOS	5/7	7/7	6/7	4/7	3/7
C	Control	8/8	8/8	8/8	7/8	8/8
	Polydextrose	8/8	8/8	8/8	4/8	6/8

other two control groups (from experiments B and C), probably due to slight variations between batches of animals (Table 3).

3.4. In vitro fermentation

Large differences among the three investigated *L. monocytogenes* strains (7291, 4446 and ScottA) were observed with respect to their ability to ferment the tested carbohydrates (Table 4). Common traits were that all three strains grew on glucose and GOS. Growth of a given strain correlated with a decrease in pH, except in the case of growth of *L. moncytogenes* 4446 on apple pectin, which was not accompanied by a pH decline, and incubation of *L. monocytogenes* ScottA with polydextose, which resulted in a pH decline indicating metabolic activity even in the absence of growth (Table 4).

3.5. L. monocytogenes specific antibodies and acute phase proteins

The level of acute phase protein in the blood is used as a marker of infection in vertebrates (Malle and De Beer, 1996). The blood levels of the acute phase protein SAA were significantly increased from pre infection to post infection in all groups (data not shown). However, no significant difference between the feeding groups was observed in the SAA increase from pre infection to post infection (data not shown).

No difference between specific antibodies against *Listeria* before and after infection on Days 4 and 7 was observed (data not shown). This was not unexpected since *Listeria* is an intracellular pathogen and therefore rarely creates a fast increases in antibody production (Smith et al., 2003).

4. Discussion

The present study shows for the first time that different non-digestible carbohydrates have entirely different effects on the intestinal colonisation and translocation of pathogenic bacteria. We compared the effects of five non-digestible dietary carbohydrates on the intestinal colonisation and translocation of the pathogenic bacterium *L. monocytogenes* in a guinea pig model.

The non-digestible carbohydrates XOS and GOS both significantly decreased the numbers of intestinal *L. monocytogenes* in ileal samples from guinea pigs (Fig. 1), although this was not reflected in faecal samples. The lower ileal counts might explain the significantly lower numbers of translocated *L. monocytogenes* in liver and spleen of animals fed with these carbohydrates (Fig. 1).

It has been suggested that non-digestible carbohydrates increase resistance to intestinal infections by selective stimulation of the indigenous bifidobacteria and lactobacilli, which are believed to elicit a multiplicity of inhibitory effects against pathogens (Orrhage and Nord, 2000). Several *in vitro* fermentation studies have shown that many lactobacilli and bifidobacteria strains grow on both GOS and XOS and thereby produce lactic acid and acetic acid (Palframan et al., 2003; Boesten and de Vos, 2008; Pan et al., 2009; Hernot et al., 2009). This could explain the observation that XOS significantly increases the relative caecal weight and decreases caecal pH (Table 2). However, a similar effect in caecum was not seen in animals fed a diet supplemented with GOS. One explanation could be that indigenous bacteria other than lactobacilli and bifidobacteria immediately use the newly produced lactic and acetic acids for fermentation. *In vitro* fermentation studies revealed that all three strains of *L. monocytogenes* grew on GOS, while only *L. monocytogenes* 4446 grew on XOS (Table 4). However, this did not result in a different distribution of the three strains *in vivo* in the XOS-fed guinea pigs than observed in the corresponding control group (data not shown).

Opposite to what was observed for XOS and GOS, feeding with inulin and apple pectin significantly increased the amount of *L. monocytogenes* detected in the liver and spleen of the infected guinea pigs (Fig. 1). However, no increased levels of *L. monocytogenes* were observed in ileum and mesenteric lymph nodes of animals fed a diet supplemented with either inulin or apple pectin. In caecum samples of inulin-fed animals, an increase was observed, while in caecum samples from animals fed a diet supplemented with apple pectin no increase in the level of *L. monocytogenes* was found. In this context, it should be noted that both *L. monocytogenes* 4446 and ScottA (but not 7291) were able to ferment inulin and apple pectin *in vitro* in monoculture (Table 4). The increase of *L. monocytogenes* counts in organs combined with the absence of an increase in the intestinal numbers suggests that other factors than the numbers of *L. monocytogenes* present in the intestinal lumen influenced the translocation of this invasive pathogen.

Only one previous study examined the effect of prebiotics on the infectivity of *L. monocytogenes* (Buddington et al., 2002). After 6 weeks on a diet supplemented with either inulin or oligofructose, mice were given a systemic infection with *L. monocytogenes* by intraperitoneal injection. Contrary to our observations, feeding with inulin or oligofructose induced a significant decrease in mortality. The Buddington study is, however, not directly comparable to our study or the common route of infection in humans due to the different animal model used, and the different infection route bypassing the gastrointestinal tract. Buddington and coworkers speculate that the mechanistic basis for the increased resistance may be an enhancement of the immune response caused by changes in the intestinal bacterial composition (Buddington et al., 2002). Although we did not measure a rise in specific antibodies against *Listeria* on Days 4 and 7 post infection, we cannot exclude that the effects of the different carbohydrates on the *L. monocytogenes* infections seen in our study can be explained by an altered immune system. Four to seven days is probably too short a period to induce a specific immune response. However, the increase in acute phase protein levels post infection, indicating activation of the innate immune response, did not reveal differences between the feeding groups.

Table 4
Fermentation parameters for batch cultures of *L. monocytogenes* on different carbohydrates. For each strain the OD_{600} of the blank sample (media without added carbohydrate grown in 24 h) were subtracted from the OD_{600} obtained after 24 h of growth of the prebiotics, showing the OD_{600} *increase*. Opposite, the pH of the media after 24 h growth was subtracted from the pH of the blank sample also grown in 24 h, showing the pH *decline*. Asterisks and grey shading indicate that the OD_{600} was significantly larger or pH significantly decreased in the sample supplemented with the carbohydrate compared to the blank sample. *: $P<0.05$; **: $P<0.005$; ***: $P<0.001$.

	L. monocytogenes 7291		*L. monocytogenes* 4446		*L. monocytogenes* ScottA	
	Growth	pH decline	Growth	pH decline	Growth	pH decline
Glucose	1.2 (±0.43)***	4.0 (±0.06))*	1.1 (±0.06)***	3.9 (±0.04)***	0.81 (±0.1)***	3.2 (0.04)***
Inulin	−0.02 (±0.1)	0.75 (±0.19)	0.53 (±0.06)***	1.3 (±0.04)***	0.64 (±0.18)**	1.0 (±0.16)**
Apple pectin	0.11 (±0.19)	0.5 (±0.08)	0.68 (±0.20)**	0.58 (±0.07)	0.66 (±0.10)**	0.65 (±0.04)***
XOS	0.27 (±0.1)	0.37 (±0.14)	0.28 (±0.17)**	0.76 (±0.02)**	0.00 (±0.15)	0.35 (±0.11)
GOS	1.1 (±0.21)***	3.1 (±0.03)*	0.63 (±0.14)*	2.7 (±0.31)**	0.92 (±0.25)***	2.3 (±0.23)***
Polydextrose	0.32 (±0.22)	0.95 (±0.03)	0.4 (±0.11)**	1.1 (±0.1)***	0.18 (±0.12)	0.92 (±0.07)**

Other studies have shown that the prebiotic carbohydrates inulin, fructooligosaccharides (FOS) and lactulose increase translocation of pathogens over the intestinal epithelium (Bovee-Oudenhoven et al., 2003; Ten Bruggencate et al., 2004; Ten Bruggencate et al., 2005; Barrat et al., 2008). It has been speculated that organic acids and other metabolites produced during the fermentation of prebiotics in the intestine may lead to irritation and impairment of the mucosal barrier and thereby enhance the translocation of pathogens (Ten Bruggencate et al., 2003). Inulin and apple pectin did not decrease caecal pH in the present study (Table 2), but that does not rule out that metabolites from the fermentation might impair the mucosal barrier. In general, only modest changes in pH were recorded in the present study (Table 2).

The *in vitro* fermentation revealed a great variability among the ability of the three *L. monocytogenes* strains to ferment the carbohydrates tested (Table 4). No connection was seen between the ability of the strains to ferment the carbohydrates *in vitro* and the infectivity of the strain in the guinea pig model, underlining that the impact of *in vitro* monoculture fermentation abilities on the *in vivo* situation should be interpreted with care. However, we want to stress that the *in vitro* observations shows that the interaction between a given (pathogenic) bacteria and a given carbohydrate may be very strain-specific, thereby underlining the relevance of animal models as the one applied here, allowing to test more than one pathogenic strain at a time.

Non-digestible carbohydrates with different chain lengths are believed to be fermented at different rates and at different sites in the intestinal tract. Both XOS and GOS have a low DP of 2–6, which makes them partly accessible for fermentation already in the small intestine where *L. monocytogenes* primarily enters the body, while carbohydrates with a high DP, like inulin, apple pectin and polydextrose are not fermented until they reach the colon (Bosscher et al., 2006). Studies have shown that the ability of short-chain carbohydrates (DP 2–10) to stimulate growth of bifidobacteria appears to be almost an order of magnitude higher than that of a carbohydrates with a higher DP (DP>10)(Roberfroid et al., 1998). We speculate that different intestinal fermentation sites for the carbohydrates tested might explain the diverse effects that are seen on the intestinal colonisation and translocation of *L. monocytogenes* in this study. However, the mechanistic basis for the difference in resistance provided by the five non-digestible carbohydrates remains to be further elucidated.

Acknowledgements

We thank Bodil Madsen and Kate Vibefeldt for excellent technical assistance and Anne Ørngreen and her department for professional handling of the animals. Obipektin AG, Lenzing AG and Danisco Limited (UK) are thankfully acknowledged for providing the carbohydrates as described in the Materials and methods section. The study was supported by a grant from The Danish Council for Strategic Research to TRL.

References

Andersen, J.B., Roldgaard, B.B., Lindner, A.B., Christensen, B.B., Licht, T.R., 2006. Construction of a multiple fluorescence labelling system for use in co-invasion studies of *Listeria monocytogenes*. BMC Microbiology 6, 86.

Andersen, J.B., Roldgaard, B.B., Christensen, B.B., Licht, T.R., 2007. Oxygen restriction increases the infective potential of *Listeria monocytogenes* in vitro in Caco-2 cells and in vivo in guinea pigs. BMC Microbiology 7, 55.

Barrat, E., Michel, C., Poupeau, G., vid-Sochard, A., Rival, M., Pagniez, A., Champ, M., Darmaun, D., 2008. Supplementation with galactooligosaccharides and inulin increases bacterial translocation in artificially reared newborn rats. Pediatric Research 64, 34–39.

Boesten, R.J., de Vos, W.M., 2008. Interactomics in the human intestine: lactobacilli and bifidobacteria make a difference. Journal of Clinical Gastroenterology 42 (Suppl 3 Pt 2), S163–S167.

Bosscher, D., Loo, J.V., Franck, A., 2006. Inulin and oligofructose as prebiotics in the prevention of intestinal infections and diseases. Nutrition Research Reviews 19, 216–226.

Bovee-Oudenhoven, I.M., Ten Bruggencate, S.J., Lettink-Wissink, M.L., Van der, M.R., 2003. Dietary fructo-oligosaccharides and lactulose inhibit intestinal colonisation but stimulate translocation of salmonella in rats. Gut 52, 1572–1578.

Buddington, K.K., Donahoo, J.B., Buddington, R.K., 2002. Dietary oligofructose and inulin protect mice from enteric and systemic pathogens and tumor inducers. Journal of Nutrition 132, 472–477.

Drider, D., Fimland, G., Hechard, Y., McMullen, L.M., Prevost, H., 2006. The continuing story of class IIa bacteriocins. Microbiology and Molecular Biology Reviews 70, 564–582.

Fonnesbech, B., Frokiaer, H., Gram, L., Jespersen, C.M., 1993. Production and specificity of poly- and monoclonal antibodies raised against *Shewanella putrefaciens*. Journal of Applied Bacteriology 74, 444–451.

Gibson, G.R., Wang, X., 1994. Regulatory effects of bifidobacteria on the growth of other colonic bacteria. Journal of Applied Bacteriology 77, 412–420.

Gibson, G.R., Beatty, E.R., Wang, X., Cummings, J.H., 1995. Selective stimulation of bifidobacteria in the human colon by oligofructose and inulin. Gastroenterology 108, 975–982.

Heegaard, P.M., Ebersbach, T., Licht, T.R., in preparation. Characterization of serum amyloid A as an acute phase protein in the guinea pig.

Hernot, D.C., Boileau, T.W., Bauer, L.L., Middelbos, I.S., Murphy, M.R., Swanson, K.S., Fahey Jr., G.C., 2009. In vitro fermentation profiles, gas production rates, and microbiota modulation as affected by certain fructans, galactooligosaccharides, and polydextrose. Journal of Agricultural and Food Chemistry 57, 1354–1361.

Kaplan, H., Hutkins, R.W., 2000. Fermentation of fructooligosaccharides by lactic acid bacteria and bifidobacteria. Applied and Environmental Microbiology 66, 2682–2684.

Larsen, C.N., Norrung, B., Sommer, H.M., Jakobsen, M., 2002. In vitro and in vivo invasiveness of different pulsed-field gel electrophoresis types of *Listeria monocytogenes*. Applied and Environmental Microbiology 68, 5698–5703.

Lecuit, M., Vandormael-Pournin, S., Lefort, J., Huerre, M., Gounon, P., Dupuy, C., Babinet, C., Cossart, P., 2001. A transgenic model for listeriosis: role of internalin in crossing the intestinal barrier. Science 292, 1722–1725.

Lomax, A.R., Calder, P.C., 2008. Prebiotics, immune function, infection and inflammation: a review of the evidence. British Journal of Nutrition 1–26.

Malle, E., De Beer, F.C., 1996. Human serum amyloid A (SAA) protein: a prominent acute-phase reactant for clinical practice. European Journal of Clinical Investigation 26, 427–435.

Mandalari, G., Nueno, P.C., Tuohy, K., Gibson, G.R., Bennett, R.N., Waldron, K.W., Bisignano, G., Narbad, A., Faulds, C.B., 2007. In vitro evaluation of the prebiotic activity of a pectic oligosaccharide-rich extract enzymatically derived from bergamot peel. Applied Microbiology and Biotechnology 73, 1173–1179.

McDonald, T.L., Weber, A., Smith, J.W., 1991. A monoclonal antibody sandwich immunoassay for serum amyloid A (SAA) protein. Journal of Immunological Methods 144, 149–155.

Orrhage, K., Nord, C.E., 2000. Bifidobacteria and lactobacilli in human health. Drugs Under Experimental and Clinical Research 26, 95–111.

Palframan, R.J., Gibson, G.R., Rastall, R.A., 2003. Carbohydrate preferences of Bifidobacterium species isolated from the human gut. Current Issues in Intestinal Microbiology 4, 71–75.

Pan, X., Wu, T., Zhang, L., Cai, L., Song, Z., 2009. Influence of oligosaccharides on the growth and tolerance capacity of lactobacilli to simulated stress environment. Letters in Applied Microbiology 48, 362–367.

Rhoades, J., Manderson, K., Wells, A., Hotchkiss Jr., A.T., Gibson, G.R., Formentin, K., Beer, M., Rastall, R.A., 2008. Oligosaccharide-mediated inhibition of the adhesion of pathogenic *Escherichia coli* strains to human gut epithelial cells in vitro. Journal of Food Protection 71, 2272–2277.

Roberfroid, M., 2007. Prebiotics: the concept revisited. Journal of Nutrition 137, 830S–837S.

Roberfroid, M.B., Van Loo, J.A., Gibson, G.R., 1998. The bifidogenic nature of chicory inulin and its hydrolysis products. Journal of Nutrition 128, 11–19.

Roldgaard, B.B., Andersen, J.B., Hansen, T.B., Christensen, B.B., Licht, T.R., 2009. Comparison of three *Listeria monocytogenes* strains in a guinea-pig model simulating food-borne exposure. FEMS Microbiology Letters 291, 88–94.

Seifert, S., Watzl, B., 2007. Inulin and oligofructose: review of experimental data on immune modulation. Journal of Nutrition 137, 2563S–2567S.

Shoaf, K., Mulvey, G.L., Armstrong, G.D., Hutkins, R.W., 2006. Prebiotic galactooligosaccharides reduce adherence of enteropathogenic *Escherichia coli* to tissue culture cells. Infection and Immunity 74, 6920–6928.

Smith, M.A., Takeuchi, K., Brackett, R.E., McClure, H.M., Raybourne, R.B., Williams, K.M., Babu, U.S., Ware, G.O., Broderson, J.R., Doyle, M.P., 2003. Nonhuman primate model for *Listeria monocytogenes*-induced stillbirths. Infection and Immunity 71, 1574–1579.

Ten Bruggencate, S.J.M., Bovee-Oudenhoven, I.M.J., Lettink-Wissink, M.L.G., Van der Meer, R., 2003. Dietary fructo-oligosaccharides dose-dependently increase translocation of salmonella in rats. Journal of Nutrition 133, 2313–2318.

Ten Bruggencate, S.J., Bovee-Oudenhoven, I.M., Lettink-Wissink, M.L., Katan, M.B., Van der, M.R., 2004. Dietary fructo-oligosaccharides and inulin decrease resistance of rats to salmonella: protective role of calcium. Gut 53, 530–535.

Ten Bruggencate, S.J., Bovee-Oudenhoven, I.M., Lettink-Wissink, M.L., Van der, M.R., 2005. Dietary fructooligosaccharides increase intestinal permeability in rats. Journal of Nutrition 135, 837–842.

Tuohy, K.M., Kolida, S., Lustenberger, A.M., Gibson, G.R., 2001. The prebiotic effects of biscuits containing partially hydrolysed guar gum and fructo-oligosaccharides—a human volunteer study. British Journal of Nutrition 86, 341–348.

Vazquez-Boland, J.A., Kuhn, M., Berche, P., Chakraborty, T., Dominguez-Bernal, G., Goebel, W., Gonzalez-Zorn, B., Wehland, J., Kreft, J., 2001. Listeria pathogenesis and molecular virulence determinants. Clinical Microbiology Reviews 14, 584–640.

Vernazza, C.L., Gibson, G.R., Rastall, R.A., 2006. Carbohydrate preference, acid tolerance and bile tolerance in five strains of Bifidobacterium. Journal of Applied Microbiology 100, 846–853.

Vos, A.P., M'Rabet, L., Stahl, B., Boehm, G., Garssen, J., 2007. Immune-modulatory effects and potential working mechanisms of orally applied nondigestible carbohydrates. Critical Review in Immunology 27, 97–140.

Wang, X., Gibson, G.R., 1993. Effects of the in vitro fermentation of oligofructose and inulin by bacteria growing in the human large intestine. Journal of Applied Bacteriology 75, 373–380.

Manuscript II

Specific indigestible carbohydrates affect epithelial adhesion and virulence gene expression in *Listeria monocytogenes*

Submitted to International Journal of Food Microbiology

Specific indigestible carbohydrates affect epithelial adhesion and virulence gene expression in *Listeria monocytogenes*

Tine Ebersbach[1]*, Jens Bo Andersen[1], Anders Bergström[1], Robert W. Hutkins[2], Tine Rask Licht[1]

[1]National Food Institute, Technical University of Denmark, Mørkhøj Bygade 19, 2860 Soeborg, Denmark.

[2]Department of Food Science and Technology, University of Nebraska - Lincoln, Nebraska 68583.

Running title: Dietary carbohydrates modulate the adhesive potential of *Listeria*

Keywords: *Listeria monocytogenes*, prebiotics, adhesion, virulence expression

*Corresponding author.

National Food Institute, Technical University of Denmark, Mørkhøj Bygade 19, 2860 Soeborg, Denmark, +45 35 88 70 91, tiebe@food.dtu.dk, (no fax)

Abstract

Dietary prebiotics carbohydrates are defined by a beneficial effect on the intestinal microbiota. However, they might also have effects that are not attributed to changes in the commensal bacterial community of the gut. In a previous study, we observed that certain carbohydrates promoted *L. monocytogenes* infection in guinea pigs, while others prevented it. The purpose of the present study was to address microbiota-independent effects of the same four indigestible carbohydrates which could explain the observed effects. We investigated the ability of xylooligosaccharides (XOS), galactooligosaccharides (GOS), inulin and polydextrose to inhibit the adherence of three different strains of *L. monocytogenes* to Caco-2 cells *in vitro* as well as to affect expression of selected virulence genes in these pathogens. Mixing with XOS significantly ($P<0.05$) decreased the ability of two of the three strains of *L. monocytogenes* to adhere to the Caco-2 cells. Additionally, 2 hours incubation with XOS followed by washing of the bacteria significantly ($P<0.05$) decreased the adherence of all three strains of *L. monocytogenes* to Caco-2 cells. Expression of several *L. monocytogenes* genes known to be involved in adherence to intestinal cells (*inlA, lap, ami, iap, aut, fdpA, actA*) were affected in a strain dependent manner by the presence of these indigestible carbohydrates in the growth media. Our results show that certain dietary carbohydrates may prevent pathogenic infections through other mechanisms than a beneficial effect on the gut microbiota.

1. Introduction

Prebiotics are currently defined as selectively fermented carbohydrates that cause specific changes in the gastrointestinal microbiota, resulting in beneficial effects on host health (Gibson *et al.*, 2004). One such effect is the suppression of pathogenic infections, which is proposed to occur via one of several mechanisms. Prebiotics enrich for specific members of the intestinal community that produce anti-pathogenic compounds (Gibson and Wang, 1994;Drider *et al.*, 2006), lower the intestinal pH (Drago *et al.*, 1997), compete for nutrients, block mucosal adhesion sites, and stimulate the immune system (Rhoades *et al.*, 2008).

While these modes of action are dependent on the carbohydrate's effect on the surrounding intestinal microbiota, only few studies have addressed direct (i.e. microbiota-independent) effects of prebiotics on the infective potential of pathogens, and most have focused on gram-negative bacteria (Shoaf *et al.*, 2006;Ruas-Madiedo *et al.*, 2006;Rhoades *et al.*, 2008;Searle *et al.*, 2009). Prebiotics may have direct inhibitory effects by blocking the mucosal adhesion sites recognized by enteric pathogens. The anti-adherence effect of some prebiotics is based on the observation that these carbohydrates structurally resemble the saccharide-containing glycoproteins present on the surface of intestinal cells and to which pathogens adhere (Kunz *et al.*, 2000). Thus, the carbohydrates act as a receptor decoy, interfering with the attachment of the pathogen to the intestinal mucosa, explaining the inhibition of pathogen adhesion seen in the presence of oligosaccharides in most of these assays (Shoaf *et al.*, 2006;Ruas-Madiedo *et al.*, 2006;Rhoades *et al.*, 2008;Searle *et al.*, 2009).

We have previously shown that *L. monocytogenes* infection of guinea pigs was prevented by dietary supplementation with xylooligosaccharides (XOS) or galactooligosaccharides (GOS), but promoted by inulin and unaffected by polydextrose (Ebersbach *et al.*, 2010). The purpose of the present study was to determine whether interactions between the pathogens and the carbohydrates could explain these observations.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Three *L. monocytogenes* isolates were used: Strain 7291, serogroup 4b, isolated from a chicken pasta salad, the clinical isolate 4446, serogroup 4b, isolated from a 63-year-old female with septicaemia (Larsen *et al.*, 2002), and the clinical reference isolate ScottA, serogroup 4b. All three strains carried nalidixic acid resistance and all also carried the plasmid pJEBAN3 coding for yellow fluorescent protein (YFP) and erythromycin resistance (Andersen *et al.*, 2006). Bacteria were cultivated on brain heart infusion (BHI) agar (Oxoid) or in liquid BHI (Oxoid). When appropriate, nalidixic acid (Sigma) or erythromycin (Sigma) were used at a final concentration of 100 µg/ml or 10 µg/ml, respectively.

2.2. Prebiotic oligosaccharides

Inulin (BENEO ST-gel; DP 2-6, purity >90%) was kindly provided by Alsiano (Birkerød, Denmark), while GOS (DP 2-6, food grade purity) and polydextrose (Litesse Ultra®; average DP of 12, food grade purity) were kindly provided by Danisco (UK) Limited, Redhill, United Kingdom. XOS (DP 2-6, purity >90%) was kindly provided by Lenzing AG, Lenzing, Austria.

2.3. Caco-2 cell culture

The human colon adenocarcinoma cell-line Caco-2 was obtained from the American Type Culture Collection (LGC Promochem, Sweden; ATCC no. HTB-37) and maintained under tissue culture conditions in Dulbecco´s modified Eagle´s minimal essential medium (Gibco-BRL) supplemented with 10% fetal bovine serum (Fischer) and 100U/ml Penicillin G. The Caco-2 cells were seeded at 3 x 10^5 cells ml^{-1} in 24-well tissue culture plates with and without 12-mm diameter glass coverslips. The Caco-2 cells were fully differentiated for 14 days by changing the culture medium every 2-3 days before used in infection assays. At late confluency, these cells express both structural and functional characteristics of enterocytes present in the small intestine (Halpin *et al.*, 2009). Maintenance of cells and subsequent experiments were carried out at 37ºC in a humidified atmosphere of 95% air and 5% CO_2. The Caco-2 cells were used between passage number 15 and 25.

2.4. Mixing of *L. monocytogenes* with carbohydrates

Anaerobically-grown overnight cultures (20 mL) of each *Listeria* strain (7291, 4446 or ScottA) were harvested, washed and the pellet was resuspended in 40 ml preheated DMEM/10% FBS. 4.5 ml of the suspension was mixed with 0.5 ml of one of the four prebiotics (final prebiotic concentration 20 mg/ml) or sterile water as a control and these mixtures were immediately used in the infection assay. The inoculum size (about 3 x 10^8 cells/ml) was determined by plating diluted samples on BHI supplemented with erythromycin and nalidixic acid.

2.5. Incubation of *L. monocytogenes* with carbohydrates

1 ml of one of the four carbohydrates (final concentration 20 mg/ml) or sterile water as a control was mixed with 9 ml of an anaerobic grown *Listeria* culture (either strain 7291, 4446 or ScottA) at OD_{600} = 0.3. After two hours of further growth the culture was spun down and washed thoroughly three times. Pellet was resuspended in 10 ml preheated DMEM/10% FBS and used in the infection assay. The inoculum size (about 2 x 10^8 cells/ml) was determined as above.

2.6. Infection assay

Prior to the infection assay, Caco-2 cells were washed twice in sterile phosphate-buffered saline (PBS). Then, 0.5 ml of the previously prepared bacterial suspension was added to each well and the plates were incubated for 1 hour at 37º C in a CO_2 incubator. The wells were washed three times with PBS to remove nonadhered *Listeria* before 0.1% Triton X-100 were added for 20 min. Serial dilutions and plating on BHI supplemented with erythromycin and nalidixic acid were used to determine the number of adhered and invaded *Listeria*. Experiments were performed in quadruplicate and repeated four times.

2.7. Confocal microscopy

Cells seeded on glass coverslips were after incubation with *Listeria* washed three times in PBS and fixed in 4% paraformaldehyde. After 20 min the cells were washed twice, dried and mounted on glass slides. Cells were observed under an inverted Confocal Microscope (Olympus IX 81)

with the 100 x objective. Representative pictures were taken by a person with no knowledge about the origin of the samples (with or without XOS).

2.8. Sample preparation and isolation of RNA for qPCR

One ml of one of the four carbohydrates (final concentration 20 mg/ml) or sterile water as control was mixed with 9 ml of each strain of anaerobically grown *Listeria* (OD_{600} = 0.3). After two hours of further growth, messenger RNA in the bacterial cultures was stabilized by addition of RNA*later* RNA Stabilization Reagent (Qiagen, Copenhagen, Denmark). Total RNA was subsequently isolated using the RNeasy mini kit (Qiagen, Copenhagen, Denmark) according to manufactures instructions. To ensure RNA samples were completely free of genomic DNA, all RNA samples were DNAse treated as outlined in the protocol of the TURBO DNA-*free*™ kit (Ambion, Applied Biosystems, Naerum, Denmark). After DNAse treatment, total RNA yields were determined using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington DE 19810, USA) and RNA quality (integrity) was verified by electrophoresis on a 2100 Bioanalyzer (Agilent Technologies, Naerum, Denmark) using an RNA 6000 Nano Chip (Agilent Technologies, Naerum, Denmark).

2.9. Quantitative Real-Time PCR (qPCR)

The mRNA transcripts of seven genes were quantified using qPCR. Primers were designed using Primer Blast (NCBI) (Table 1). Quantitative PCR amplification was performed in a ABI 7900 Real-Time PCR system (Applied Biosytems, Naerum, Denmark) using the EXPRESS SYBR® GreenER™ qPCR Supermix with Premixed ROX kit (Invitrogen A/S, Taastrup, Denmark). Aliquots of 200 ng Total RNA of each sample were reverse transcribed into cDNA using the SuperScript® VILO™ cDNA synthesis kit and the protocol outlined by the manufacturer (Invitrogen A/S, Taastrup, Denmark). Two µL of a 1:100 dilution of the cDNA product was added to 18 uL solution containing EXPRESS SYBR® GreenER™ qPCR Supermix with Premixed ROX kit (Invitrogen A/S, Taastrup, Denmark) and 200 nM of the forward and reverse primers each.

Amplification was initiated at 95°C for 10 minutes followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. A melting-curve analysis between 60°C and 95°C was performed after each PCR

to check the specificity of the amplification product. This PCR program was found by preliminary experiments on target DNA in order to optimize reaction parametres and primer concentrations. The program was efficient and consistent for all primers used. During the preliminary experiments, the amplification products were further subjected to gel electrophoresis in 2% agarose, followed by ethidium bromide staining to verify correct amplicon sizes. All samples were measured in either duplicate or triplicate. Control PCR´s were included to detect background contamination (no-template control) and to verify that there was no contaminating chromosomal DNA (DNAse treated Total RNA sample). In order to compare the expression level of each gene across all tested conditions, all expression levels were normalised to the expression level of housekeeping gene reference values. The geometric mean of the reference values for the *Eubacteria* 16S gene and the *gap* gene (lmo2459) of *L. monocytogenes* were used as housekeeping reference gene for these calculations as suggested by Vandesompele *et al* (2002). mRNA quantification was performed in triplicate from RNA extracted from three independent cultures.

2.10. Statistical analysis

Statistical analysis of the adhesions assays were performed using the statistical software in Microsoft Office Excel XP (Microsoft, USA). A one-way analysis of variance was used to determine the effect of the non digestible carbohydrates on the number of *Listeria*/Caco-2 cell. Tests were considered statistically significant if P values <0.05 were obtained.

Statistical analysis of quantitative PCR data was performed with SAS JMP version 7.0. After graphical verification of the normality assumption, data was analyzed by one-way ANOVA followed by a pair-wise multiple comparison of means (Student's t). Tests were considered statistically significant if P values <0.05 were obtained.

3. Results

3.1. Effect of mixing with carbohydrates on the adherence of *L. monocytogenes* to Caco-2 cells

The addition of XOS to the bacterial culture immediately before addition of the suspension to the Caco-2 cells significantly ($P<0.05$) reduced the numbers of *L. monocytogenes* 7291 and ScottA adhered or invaded into the Caco-2 cells (Figure 1). Confocal microscopy was used for visual inspection of bacteria and Caco-2 cells (Figure 2), and confirmed an apparent lower number of adhered bacteria in the presence of XOS. Addition of GOS, inulin or polydextrose had no significant effect on the ability of the three strains to adhere to or invade the Caco-2 cells (Figure 1).

3.3. Effect of incubation with carbohydrates on the adherence of *L. monocytogenes* to Caco-2 cells

Incubation with XOS for two hours, followed by washing of the bacteria, significantly reduced the number of adhered and invaded bacteria per Caco-2 cell for all three strains of *L. monocytogenes* ($P<0.05$ for strain 7291 and 4446, $P<0.001$ for ScottA). A similar effect was not observed for the other carbohydrates (Figure 3).

3.2. Effect of incubation with carbohydrates on expression of *L. monocytogenes* genes potentially involved in adherence

There was no significant difference in growth OD after two hours incubation between samples incubated with different carbohydrates.

The effect of incubating the cells with the carbohydrates on the expression pattern of seven adherence-related genes was comparable for *L. monocytogenes* strains 4446 and ScottA, while the expression profile of strain 7291 was quite different (Figure 4). Several genes were affected in 4446 and ScottA, while only one gene (*iap*) was affected in 7291. Most of the affected genes were down-regulated by carbohydrate incubation except for *iap,* which was up-regulated by

inulin and XOS in all three strains, and *lap*, which was up-regulated by polydextrose in 4446 and ScottA (Figure 4).

4. Discussion

We previously reported that four non-digestible carbohydrates had different effects on the infective potential of three strains of *L. monocytogenes* in guinea pigs (Ebersbach *et al.*, 2010).

In this study, our goal was to investigate the mechanisms accounting for these observations using the same strains of *L. monocytogenes* and the same carbohydrates. We found that XOS significantly inhibited the adhesion of two out of three *L. monocytogenes* strains to Caco-2 tissue culture cells when added immediately prior to the start of the infection assay (Figure 1 and 2). Additionally, two hours incubation with XOS followed by removal of the carbohydrate reduced the adhesion of all three *L. monocytogenes* strains (Figure 3). This suggests that our previous observation that dietary XOS reduces the severity of *Listeria* infections *in vivo* (Ebersbach *et al.*, 2010) may be explained by an inhibition of pathogen attachment to the guinea pig epithelium *in vivo*. However, while our earlier study reported that GOS prevented Listerial infection in guinea-pigs (Ebersbach *et al.*, 2010), no effect of this carbohydrate on *in vitro* adhesion to Caco-2 cells was observed (Figure 1 and 3). Therefore, different mechanisms may be responsible for the preventive effects observed *in vivo* for these two types of oligosaccharides. Inulin, which increased the severity of the infection in guinea pigs (Ebersbach *et al.*, 2010), had no effect on Listerial adhesion to epithelial cells lines in the present study (Figure 1 and 3). Polydextrose had neither effect *in vivo* nor *in vitro*.

Our observations with XOS (Figure 1 and 3) is consistent with previous reports showing that specific oligosaccharides are able to inhibit adherence of gram-negative bacteria to intestinal tissue culture cells (Lee and Puong, 2002;Shoaf *et al.*, 2006;Rhoades *et al.*, 2008;Searle *et al.*, 2009). According to the anti-adherence model, these oligosaccharides act as receptor decoys for pathogens by mimicking the host cell receptor on the intestinal cell, to which the pathogen adheres before internalization. By binding to the oligosaccharide instead of to the intestinal cells, the pathogen is washed from the gastrointestinal tract and does not cross the epithelial barrier.

Strain 4446 is the only one of the three *L. monocytogenes* strains, that ferments XOS (Ebersbach *et al.*, 2010), and additionally the only one for which adhesion is not blocked by mixing with XOS (Figure 1). We speculate that because strain 4446 metabolizes XOS, it is taken up by the bacteria instead of sticking to the cell surface and blocking adherence to the Caco-2 cells.

However, it should be noted that inhibition of adherence of strain 4446 was seen after incubation with XOS (Figure 3), suggesting that this oligosaccharide blocks Listerial adhesion through other mechanisms than direct interaction with the bacterial surface.

It is well known that carbohydrates influence expression of virulence genes in *L. monocytogenes,* either because the carbohydrate functions as a signaling molecule *per se* or more likely as a result of catabolite repression (Milenbachs *et al.*, 1997;Gilbreth *et al.*, 2004). This led us to investigate if incubation with different carbohydrates affected expression of genes known to be involved in the infection and internalization pathways in *L. monocytogenes*.

The effect on adherence gene expression of strain 7291 was markedly different from the effects on strain 4446 and ScottA, which were almost identical (Figure 4). Most of the seven genes of strain 4446 and ScottA were affected by incubation with the carbohydrates, while the only gene affected in strain 7291 was *iap* (Figure 4A). However, this different expression profile of strain 7291 was not reflected in the *in vitro* adherence assay (Figure 3). While only XOS reduced the adherence of *L. monocytogenes* to the Caco-2 cells lines, all of the four prebiotics significantly down regulated the expression of *inlA* in strain 4446 and ScottA. Still, it was XOS that had the strongest effect on the expression of *inlA,* which encodes the surface protein Internalin A. Through its interaction with the host cell receptor E-cadherin (Mengaud *et al.*, 1996), Internalin A is believed to be the primarily protein involved in infection of enterocytes by *L. monocytogenes* (Lecuit *et al.*, 2001). We speculate that the expression of *inlA* would need to drop below a certain level before affecting the measured *in vitro* adherence. Indeed, expression of *inlA* is known to differ between strains of *L. monocytogenes,* and a correlation exists between the level of *inlA* expression and the invasion capacity into Caco-2 cells (Werbrouck *et al.*, 2006).

The expression of *actA* and *inlA* are positively regulated by PrfA the master virulence regulator of *L. monocytogenes* (Milohanic *et al.*, 2003), and σ^B, which positively regulates the expression of *iap* (Hain *et al.*, 2008). On the contrary, not much is known about the regulation of expression of the other four tested genes. Since many of the genes of strains 4446 and ScottA are similarly

down- or up-regulated after incubation with indigestible carbohydrates (Figure 4), it may be speculated that the expression of some of the genes is controlled by a common gene regulator.

Several autolysins have been shown to play a role in the adherence of *L. monocytogenes* to intestinal cells and their role might be to fine tune the surface architecture of *L. monocytogenes* priming the bacteria for adherence and invasion of enterocytes. The autolysins believed to be involved in adherence of *L. monocytogenes* are Ami, p60 and Auto, encoded by *ami, iap*, and *aut*, respectively (Milohanic *et al.*, 2001;Cabanes *et al.*, 2004;Faith *et al.*, 2007). While *ami* and *aut* were downregulated by one or more of the tested carbohydrates in 4446 and ScottA, *iap* was significantly up-regulated by inulin and XOS in all three Listeria strains (Figure 4). However, these effects were not reflected in the phenotypic adhesion to Caco- 2 cells (Figure 3).

Another protein that has been shown to be important for adherence of *L. monocytogenes* is the protein LAP (Listeria adhesion protein). LAP is involved in binding of *L. monocytogenes* to several human intestinal cell lines via interaction with its receptor Hsp60 (Heat shock protein 60). *L. monocytogenes* also has the ability to bind to cell-surface fibronectin through FbpA (fibronectin-binding protein A) which has been shown to be important for oral infection of transgenic mouse (Dramsi *et al.*, 2004). Expression of LAP was seen to be significantly down-regulated by incubation with XOS, but up-regulated by incububation with polydextrose in strains 4446 and Scott A, which may contribute to the observed inhibitory effect of XOS on *in vitro* adherence (Figure 3) as well as on *in vivo* infection (Ebersbach *et al.*, 2010).

The surface protein ActA is a major virulence factor of *L. monocytogenes,* mediating actin-based intra- and intercellular spread (Smith *et al.*, 1995). In addition, the ability of *L. monocytogenes* to adhere to intestinal cell lines is significantly impaired if ActA is deleted (Suarez *et al.*, 2001). Furthermore, expression of ActA enables *L. innocua* to invade epithelial cells, suggesting that ActA might also play a role in the intestinal adherence of *L. monocytogenes* (Suarez *et al.*, 2001). Expression of ActA was significantly reduced in strain 4446 by three out of four carbohydrates, and in strain ScottA by all of the tested carbohydrates.

Collectively, our results suggest that dietary carbohydrates may alter the adhesive and infective potential of *L. monocytogenes* present in the intestine through direct sticking to the bacterial surface, as well as through effects on expression of adhesion-related genes. In particular, XOS had a significant reductive effect on the adhesion of *L. monocytogenes* to epithelial cells *in vitro*,

which may explain the previously reported preventive effect of this oligosaccharide on *in vivo* infection (Ebersbach *et al.*, 2010).

Acknowledgements

We thank Bodil Madsen, Kate Vibefeldt and Terry Fangman for excellent technical assistance. Lenzing AG (AT), Danisco Limited (UK) and Alsiano (DK) are thankfully acknowledged for providing the carbohydrates as described in the Materials and Methods section. The study was supported by The Danish Council for Strategic Research through a grant (21-06-0026) given to Tine Rask Licht.

Reference List

Andersen,J.B., Roldgaard,B.B., Lindner,A.B., Christensen,B.B., and Licht,T.R. (2006). Construction of a multiple fluorescence labelling system for use in co-invasion studies of Listeria monocytogenes. BMC.Microbioloy. *6*, 86.

Cabanes,D., Dussurget,O., Dehoux,P., and Cossart,P. (2004). Auto, a surface associated autolysin of Listeria monocytogenes required for entry into eukaryotic cells and virulence. Molecular Microbiology. *51*, 1601-1614.

Drago,L., Gismondo,M.R., Lombardi,A., de,H.C., and Gozzini,L. (1997). Inhibition of in vitro growth of enteropathogens by new Lactobacillus isolates of human intestinal origin. FEMS Microbiolology Letters. *153*, 455-463.

Dramsi,S., Bourdichon,F., Cabanes,D., Lecuit,M., Fsihi,H., and Cossart,P. (2004). FbpA, a novel multifunctional Listeria monocytogenes virulence factor. Molecular Microbiology. *53*, 639-649.

Drider,D., Fimland,G., Hechard,Y., McMullen,L.M., and Prevost,H. (2006). The continuing story of class IIa bacteriocins. Microbiology Molecular Biology Reviews. *70*, 564-582.

Ebersbach,T., Jørgensen,J.B., Heegaard,P.M., Lahtinen,S.J., Ouwehand,A.C., Poulsen,M., Frøkiær,H., and Licht,T.R. (2010). Certain dietary carbohydrates promote Listeria infection in a guinea pig model, while others prevent it. International Journal of Food Microbiology. *140*, 218-224.

Faith,N.G., Kathariou,S., Neudeck,B.L., Luchansky,J.B., and Czuprynski,C.J. (2007). A P60 mutant of Listeria monocytogenes is impaired in its ability to cause infection in intragastrically inoculated mice. Microbial Pathogen. *42*, 237-241.

Gibson,G.R., Probert,H.M., Loo,J.V., Rastall,R.A., and Roberfroid,M.B. (2004). Dietary modulation of the human colonic microbiota: updating the concept of prebiotics. Nutrition Research Reviews. *17*, 259-275.

Gibson,G.R. and Wang,X. (1994). Regulatory effects of bifidobacteria on the growth of other colonic bacteria. Journal of Applied Bacteriology. *77*, 412-420.

Gilbreth,S.E., Benson,A.K., and Hutkins,R.W. (2004). Catabolite repression and virulence gene expression in Listeria monocytogenes. Current Microbiology. *49*, 95-98.

Hain,T. *et al.* (2008). Temporal transcriptomic analysis of the Listeria monocytogenes EGD-e sigma(B) regulon. Bmc Microbiology *8*.

Halpin,R.M., Brady,D.B., O'Riordan,E.D., and O'Sullivan,M. (2009). Untreated and enzyme-modified bovine whey products reduce association of Salmonella Typhimurium, Escherichia coli O157:H7 and Cronobacter malonaticus (formerly Enterobacter sakazakii) to CaCo-2 cells. Journal of Applied Microbiology.

Kunz,C., Rudloff,S., Baier,W., Klein,N., and Strobel,S. (2000). Oligosaccharides in human milk: structural, functional, and metabolic aspects. Annual Review of Nutrition. *20*, 699-722.

Larsen,C.N., Norrung,B., Sommer,H.M., and Jakobsen,M. (2002). In vitro and in vivo invasiveness of different pulsed-field gel electrophoresis types of Listeria monocytogenes. Journal of Applied and Environmental Microbiology. *68*, 5698-5703.

Lecuit,M., Vandormael-Pournin,S., Lefort,J., Huerre,M., Gounon,P., Dupuy,C., Babinet,C., and Cossart,P. (2001). A transgenic model for listeriosis: role of internalin in crossing the intestinal barrier. Science *292*, 1722-1725.

Lee,Y.K. and Puong,K.Y. (2002). Competition for adhesion between probiotics and human gastrointestinal pathogens in the presence of carbohydrate. British Journal of Nutrition. *88 Suppl 1*, S101-S108.

Mengaud,J., Ohayon,H., Gounon,P., Mege,R.-M., and Cossart,P. (1996). E-cadherin is the receptor for internalin, a surface protein required for entry of L. monocytogenes into epithelial cells. Cell *84*, 923-932.

Milenbachs,A.A., Brown,D.P., Moors,M., and Youngman,P. (1997). Carbon-source regulation of virulence gene expression in Listeria monocytogenes. Journal of Molecular Microbiology. *23*, 1075-1085.

Milohanic,E., Glaser,P., Coppee,J.Y., Frangeul,L., Vega,Y., Vazquez-Boland,J.A., Kunst,F., Cossart,P., and Buchrieser,C. (2003). Transcriptome analysis of Listeria monocytogenes

identifies three groups of genes differently regulated by PrfA. Journal of Molecular Microbiology. *47*, 1613-1625.

Milohanic,E., Jonquieres,R., Cossart,P., Berche,P., and Gaillard,J.L. (2001). The autolysin Ami contributes to the adhesion of Listeria monocytogenes to eukaryotic cells via its cell wall anchor. Journal of Molecular Microbiology. *39*, 1212-1224.

Rhoades,J., Manderson,K., Wells,A., Hotchkiss,A.T., Jr., Gibson,G.R., Formentin,K., Beer,M., and Rastall,R.A. (2008). Oligosaccharide-mediated inhibition of the adhesion of pathogenic Escherichia coli strains to human gut epithelial cells in vitro. Journal of Food Protection. *71*, 2272-2277.

Ruas-Madiedo,P., Gueimonde,M., de los Reyes-Gavilan CG, and Salminen,S. (2006). Short communication: effect of exopolysaccharide isolated from "viili" on the adhesion of probiotics and pathogens to intestinal mucus. Journal of Dairy Science. *89*, 2355-2358.

Searle,L.E. *et al.* (2009). A mixture containing galactooligosaccharide, produced by the enzymic activity of Bifidobacterium bifidum, reduces Salmonella enterica serovar Typhimurium infection in mice. Journal of Medical Microbiology. *58*, 37-48.

Shoaf,K., Mulvey,G.L., Armstrong,G.D., and Hutkins,R.W. (2006). Prebiotic galactooligosaccharides reduce adherence of enteropathogenic Escherichia coli to tissue culture cells. Infection and Immunity. *74*, 6920-6928.

Smith,G.A., Portnoy,D.A., and Theriot,J.A. (1995). Asymmetric distribution of the Listeria monocytogenes ActA protein is required and sufficient to direct actin-based motility. Journal of Molecular Microbiology. *17*, 945-951.

Suarez,M., Gonzalez-Zorn,B., Vega,Y., Chico-Calero,I., and Vazquez-Boland,J.A. (2001). A role for ActA in epithelial cell invasion by Listeria monocytogenes. Cellular Microbiology. *3*, 853-864.

Vandesompele,J., De,P.K., Pattyn,F., Poppe,B., Van,R.N., De,P.A., and Speleman,F. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biology. *3*.

Werbrouck,H., Grijspeerdt,K., Botteldoorn,N., Van,P.E., Rijpens,N., Van,D.J., Uyttendaele,M., Herman,L., and Van,C.E. (2006). Differential inlA and inlB expression and interaction with human intestinal and liver cells by Listeria monocytogenes strains of different origins. Applied and Environmental Microbiology. *72*, 3862-3871.

Target	Name	Sequence (5'→3')	Amplicon	Source
ActA	ActA_1F	GCTTCGGACTTCCCGCCACC	172 bp	This study
	ActA_1R	GCATTGGCGTCTCTGGCAAAGC		This study
InlA	InlA-F	CGGATAAAATGCCGACAAAT	147 bp	This study
	InlA-R	CTTTTGTTGGTGCCGTAGGT		This study
LAP	LAP-F	TCCTCACGGTCGTGCCAATGC	157 bp	This study
	LAP-R	AGTTGCAGCAGGGAAGCCGA		This study
Ami	Ami-F	AATGTCCGCAGCGGTCCTGG	141 bp	This study
	Ami-R	CCAGCTTGCAACCCAACCGC		This study
FbpA	FbpA-F	CCATCAACCGTTCTCGCATGAACTT	142 bp	This study
	FbpA-R	CATCGGTGGGGTTGCTGGGT		This study
p60	p60-F	GCGCTACTGGCCCAAGCTGT	109 bp	This study
	p60-R	GCAGAGGCATATTGTTGCTTCGC		This study
Aut	Aut-F	ACGGTTTGACAACTGTTGGCGG	133 bp	This study
	Aut-R	GCCTGGGCAGCAGGTGCTATTT		This study

Table 1: Primers used for qPCR in this study.

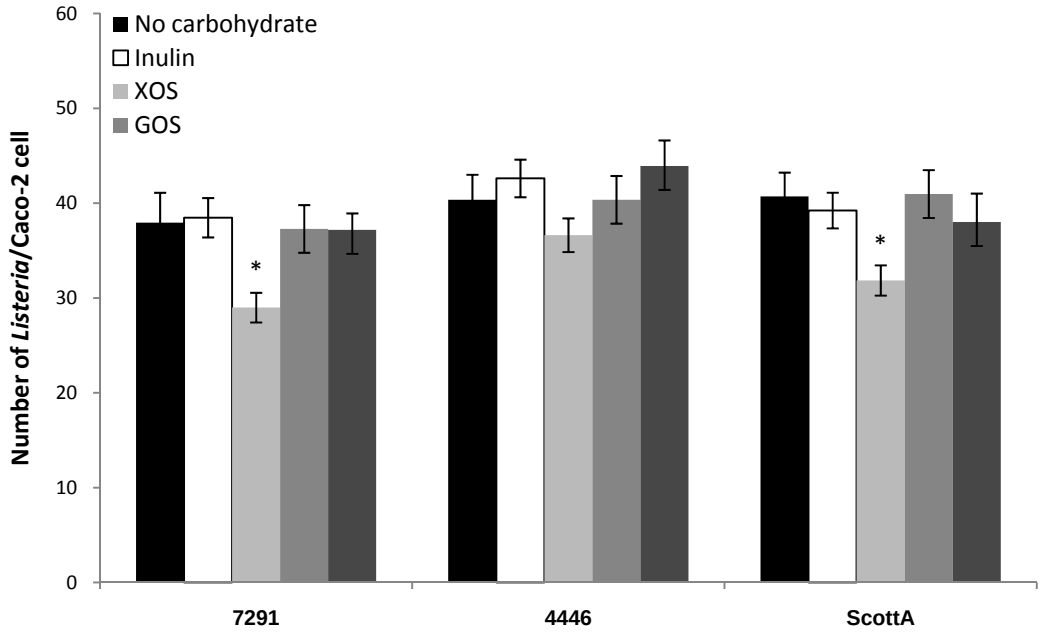


Figure 1: Inhibition of *L. monocytogenes* adhesion to Caco-2 tissue culture cells by carbohydrates. Values significantly different from the control group (no carbohydrates) are marked by stars. *: $P < 0.05$.

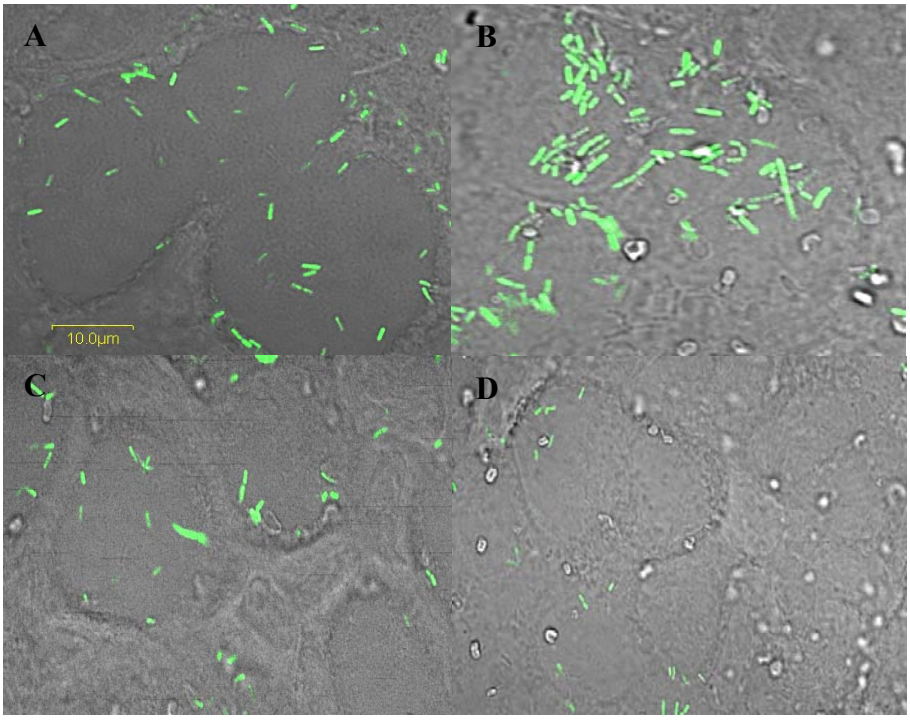


Figure 2: Confocal microscopy showing representative fields of Caco-2 cells challenged with *L. monocytogenes* strains 7291 (AC) and ScottA (BD) before (AB) and after (CD) mixing of the Listerial cells with XOS.

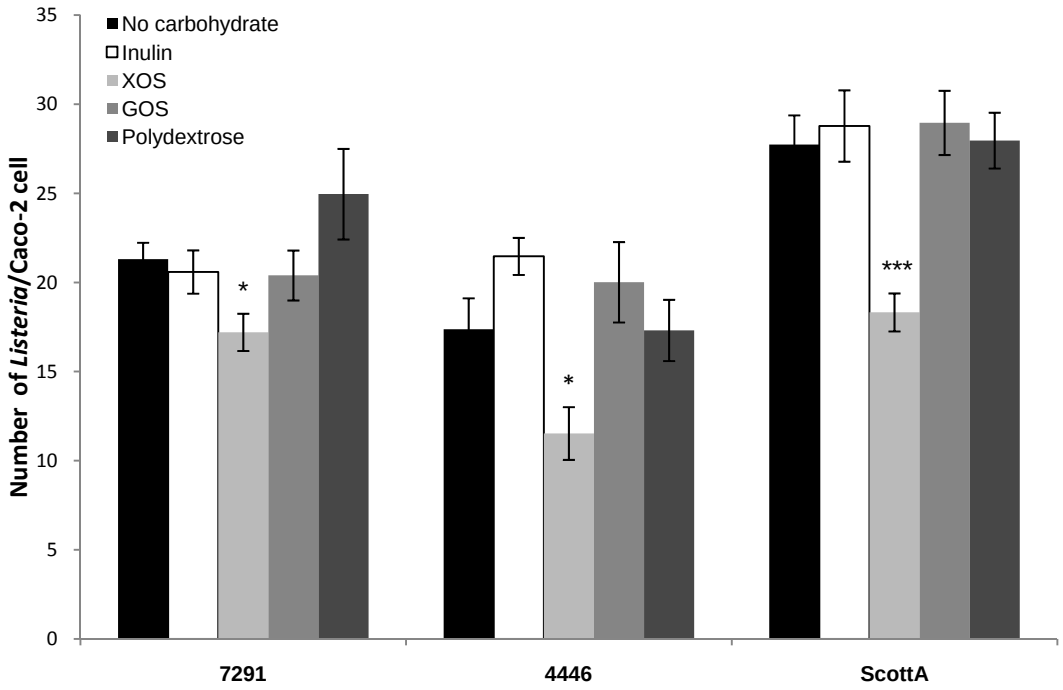
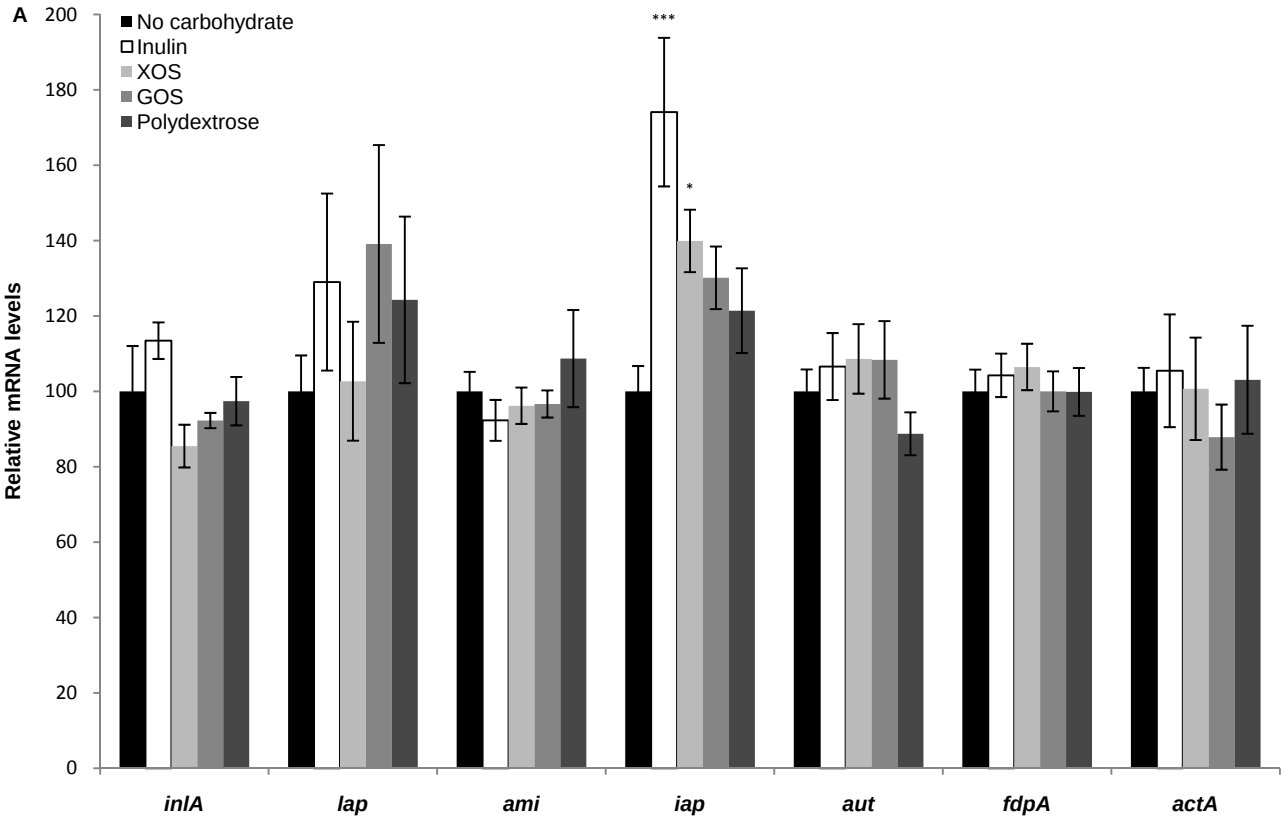
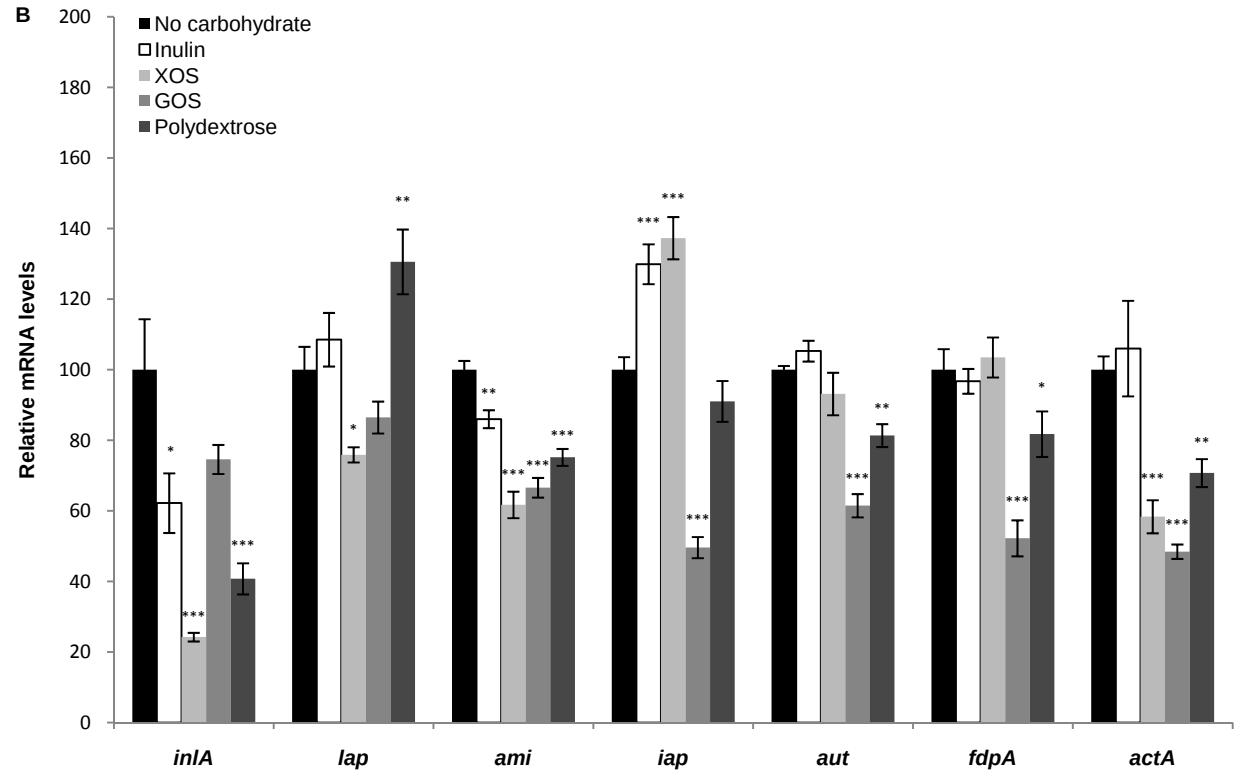


Figure 3: Inhibition of *L. monocytogenes* adhesion to Caco-2 tissue culture cells after incubation of the bacteria for two hours with the respective carbohydrates, followed by thorough washing. Values significantly different from the control group (no carbohydrate) are marked by stars. *: $P < 0.05$; *** <0.001.


A
No carbohydrate
Inulin
XOS
GOS
Polydextrose
Relative mRNA levels
0
20
40
60
80
100
120
140
160
180
200
inlA
lap
ami
iap
aut
fdpA
actA


B
No carbohydrate
Inulin
XOS
GOS
Polydextrose
Relative mRNA levels
0
20
40
60
80
100
120
140
160
180
200
inlA
lap
ami
iap
aut
fdpA
actA

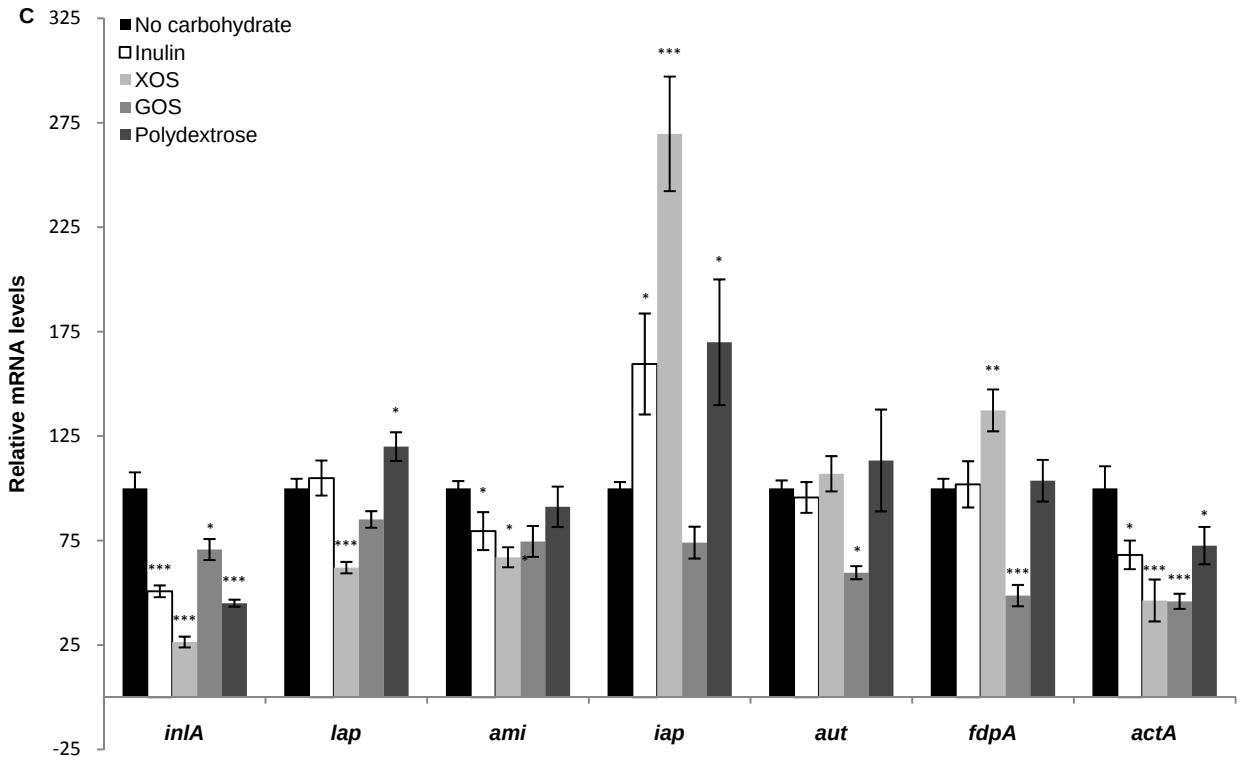


Figure 4: mRNA levels of *L. monocytogenes* strains 7291 (A)**,** 4446 (B), and ScottA (C). Results were calculated relatively as ratios to control group (no carbohydrate), which was set to 100. Values significantly different from the control group are marked by stars. *: $P < 0.05$; **: $P < 0.005$; ***: $P < 0.001$.